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EDITORIAL

Gut microbiota and liver diseases

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Abstract

Several studies revealed that gut microbiota are associated with various human diseases, *e.g.*, metabolic diseases, allergies, gastroenterological diseases, and liver diseases. The liver can be greatly affected by changes in gut microbiota due to the entry of gut bacteria or their metabolites into the liver through the portal vein, and the liver-gut axis is important to understand the pathophysiology of several liver diseases, especially non-alcoholic fatty liver disease and hepatic encephalopathy. Moreover, gut microbiota play a significant role in the development of alcoholic liver disease and hepatocarcinogenesis. Based on these previous findings, trials using probiotics have been performed for the prevention or treatment of liver diseases. In this review, we summarize the current understanding of the changes in gut microbiota associated with various liver diseases, and we describe the therapeutic trials of probiotics for those diseases.

Key words: Gut microbiota; Immune system; Liver disease; Metabolites; Non-alcoholic fatty liver disease; Hepatic encephalopathy

Core tip: Gut microbiota are associated with various human diseases (*e.g.*, metabolic, gastroenterological and liver diseases, and allergies). Genomic analyses of gut microbiota have enabled the comprehensive identification of the population of gut bacteria and revealed that changes in these populations are involved in various diseases' pathophysiology. The liver is affected by changes in the intestinal milieu due to the entry of gut bacteria or their metabolites into the liver through the portal vein. Here we summarize the current understanding of changes in gut microbiota associated with various liver diseases. We also summarize the recent therapeutic trials of probiotics in liver diseases.

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INTRODUCTION

More than 10^{14} microorganisms live in the human gastroenterological tract, including > 10^4 bacterial species. Most of the bacteria are anaerobic, and the numbers and composition of the bacteria differ according to the site of the gut (Table 1[1]). The

Table 1 Composition of gut microbiota[1]

Microorganism	Stomach	Jejunum	Ileum	Colon
Total count	$0\text{-}10^4$	$0\text{-}10^5$	$10^4\text{-}10^8$	$10^{10}\text{-}10^{12}$
Aerobic microorganisms				
Streptococcus	$0\text{-}10^3$	$0\text{-}10^4$	$10^2\text{-}10^4$	$10^3\text{-}10^5$
Enterococcus	rare	$0\text{-}10^2$	$10^2\text{-}10^4$	$10^5\text{-}10^{10}$
Staphylococcus	$0\text{-}10^3$	$0\text{-}10^3$	$10^2\text{-}10^5$	$10^4\text{-}10^6$
Enterobacteria	$0\text{-}10^2$	$0\text{-}10^3$	$10^2\text{-}10^7$	$10^4\text{-}10^{10}$
Anaerobic microorganisms				
Peptostreptococcus	$0\text{-}10^3$	$0\text{-}10^3$	$10^2\text{-}10^6$	$10^{10}\text{-}10^{12}$
Bifidobacterium	$0\text{-}10^2$	$0\text{-}10^4$	$10^3\text{-}10^9$	$10^8\text{-}10^{11}$
Lactobacillus	$0\text{-}10^3$	$0\text{-}10^4$	$10^2\text{-}10^5$	$10^6\text{-}10^8$
Clostridium	rare	rare	$10^2\text{-}10^4$	$10^6\text{-}10^9$
Eubacterium	rare	rare	rare	$10^9\text{-}10^{12}$
Veillonella	$0\text{-}10^2$	$0\text{-}10^3$	$10^2\text{-}10^4$	$10^3\text{-}10^6$
Fusobacterium	$0\text{-}10^2$	$0\text{-}10^3$	$10^3\text{-}10^4$	$10^6\text{-}10^8$
Bacteroides fragillis	rare	$0\text{-}10^3$	$10^3\text{-}10^7$	$10^{10}\text{-}10^{12}$
Prevotella	$0\text{-}10^2$	$10^2\text{-}10^4$	$10^3\text{-}10^4$	$10^4\text{-}10^5$

numbers of bacteria increase from the stomach, to the jejunum, ileum and colon. The composition of the bacterial flora also changes according to age and diet[2,3]. It has been clarified that gut microbiota play a critical role in the formation of the gut immune system[4,5] and that they also affect the systemic immune system[6,7]. Moreover, interactions between gut microbiota and the liver[8-12] or brain[13-15] have been analyzed. The composition of the microbiota is also associated with various diseases. In this review, we summarize the current understanding of the role of gut microbiota, especially in relation to liver diseases, and we describe relevant clinical trials of the treatment of liver diseases by probiotics.

COMPOSITION OF GUT MICROBIOTA

Methods for analyzing gut microbiota

Since most of the gut bacteria cannot be cultured, comprehensive analyses of them have been difficult[16]. Advances in molecular biological techniques have enabled the analyses of the whole genomes of gut microbiota, and real-time PCR, microarray, and pyrosequencing have been applied to improve the resolution of the microbial biodiversity and quantification of microbial species[17]. Among those gene-based methods, DNA pyrosequencing based on 16S rRNA genes[18] is currently the most useful method for comprehensive analysis of gut microbiota with high resolution and accurate quantification.

Composition of gut microbiota and its change according to age

Claesson *et al*[19] analyzed the composition of intestinal microbiota by pyrosequencing of over 40000 16S rRNA gene V4 region amplicons. They found that the phylum *Bacteroides* was the most predominant species of bacteria in 68% of the individuals studied, with an average proportion of 57%, followed by the phylum *Firmicutes* with an average proportion of 40%. Other detected species were *Proteobacteria*, *Actinobacteria*, and *Faecalibacteria.* The composition differed dramatically among individuals, and was also different according to the individuals' ages. Elderly individuals were reported to show increased *Bacteroides* species diversity whereas *bifidobacteria* were reduced[20], although contrasting results have been obtained[21,22].

FUNCTIONS OF GUT MICROBIOTA

Digestion and metabolism

Human enzymes are known to be unable to digest complex carbohydrates and plant polysaccharides. Instead, gut microbiota ferment the non-digestible carbohydrates, including cellulose, resistant starch and inulin in the colon to yield energy for microbial growth and end products such as short-chain fatty acids (SCFAs)[23]. The SCFAs formed are organic fatty acids, and the major SCFAs consist of acetate, propionate and butyrate. Butylate is an energy substrate for the colonic epithelium, and acetate and propionate are energy substrates for peripheral tissues[23]. Propionic and butyric acids were shown to regulate cell proliferation and differentiation, and to induce hormone release[24], the hepatic control of lipids, and carbohydrate metabolism[25]. Moreover, several SCFAs have been shown to exert anti-inflammatory and immunomodulatory effects[26,27].

Immune response to gut microbiota

Although we harbor more than 10^4 bacterial species in the gut and the composition is relatively stable in each person without causing inflammation[28], the mechanisms responsible for maintaining the flora over the long term are not yet clearly understood. The maintenance of the gut flora may be based on immune tolerance to microbiota because of the formation of the repertoire of microbiota during early life after birth, when the immune system is too immature to eradicate intestinal micro-organisms.

Round *et al*[29] showed that *Bacteroides fragilis* could induce immune tolerance to gut microbiota by developing and promoting functions of Foxp3+ regulatory T cells (Tregs) through polysaccharide A produced by the bacteria. In another study, certain *Clostridium* species, especially phylogenetic clusters IV and XIV (but not *Lactobacillus* or *Bacteroides* species) were the most effective in inducing the differentiation of Tregs[30]. Natural killer T (NKT) cells, a subgroup of T cells that recognize self-antigens and microbial lipid antigens presented by CD1d, have an important role in the development and the composition of gut microbiota through the CD1d molecule. Conversely, the development and maturation of mucosal and systemic NKT cells are controlled by commensal gut microbiota[31]. Moreover, CD1d presents self-antigens and microbial lipid antigens to NKT cells, which are

Table 2 Changes in gut microbiota in human diseases[35]

Diseases	Change in microbiota
Allergies	*Lactobacillus* spp. ↓
	Bifidbacterium adolescentis ↓
	Clostridium difficile ↓
	Helicobacter pylori ↓
Autism	*Bacteroidetes* ↑
	Proteobacteria ↑
	Actinobacteria ↓
	Firmicutes ↓
Obesity	*Bacteroidetes* ↓
	Lactobacillus ↑
	Firmicutes/Bacteroidetes ratio ↓
Type 2 Diabetes	*Firmicutes* ↓
	Clostridia ↓
	Betaproteobacteria ↓
	Bacteroidetes/Firmicutes ratio ↑
Celiac disease	*Bacteroides vulgatus* ↑
	Escherichia coli ↓
	Clostridium coccoides ↓

involved in the pathogenesis of human inflammatory bowel disease[32]. Thus, cross-talk between the microbiota and CD1d-restricted NKT cells plays an important role in microbial homeostasis and intestinal inflammation.

These overall data suggest that the immune tolerance is important not only for the maintenance of gut microbiota, but also for suppressing harmful immune responses to microbiota in the gut. Inflammatory bowel diseases are now thought to be caused by an uncontrolled immune response to gut microbiota[33].

Role of gut microbiota in the maturation of the immune system

The role of gut microbiota in gut immune maturation, including the numbers of intestinal CD4+and CD8+ T cells and dendritic cells, has been demonstrated in mice[5]. The effect of early gut colonization on systemic immune responses was also shown by Hansen *et al*[34], who reported that a single oral inoculation of a bacteria suspension made from caecal content of specific pathogen-free mice to germ-free mice at 3 wk of age, but not at 1 wk of age, permanently changed the gut microbiota composition, and the delayed colonization caused permanent changes in the immune systems of the mice. The mechanisms responsible for the influence of commensal bacteria on host immunity are thought to be nutrient- and metabolite-dependent.

Collectively, these data indicate an interplay between gut microbiota and the host's immune system.

GUT MICROBIOTA AND HUMAN DISEASES

Several specific changes in the composition of gut microbiota in a number of human diseases have been reported (Table 2[35]). It would be reasonable to speculate that not a single disease-causing microbe but rather microbial dysbiosis causes those diseases.

GUT MICROBIOTA AND THE LIVER

Since 70% of the liver's blood is supplied from the portal vein, gut-derived toxins and microbial products constantly enter the liver. The liver could thus be greatly influenced by the composition and function of gut microbes, mainly by receiving metabolites derived from the microbes.

In normal conditions, small amounts of bacteria or bacterial metabolites enter the liver, and most of them are eliminated by Kupffer cells with little activation. However, when the gut-mucosal barrier is damaged by intestinal inflammation or portal hypertension, large amounts of bacteria enter the liver and activate Kupffer cells and hepatic stellate cells. One of the pathogenic bacteria-derived factors is lipopolysaccharide (LPS), and LPS activates those cells *via* its binding to Toll-like receptor 4 on their surface. Upon the activation of those cells, pro-inflammatory cytokines are produced and are involved in liver damage. It is of note that alcohol was shown to disrupt the intestinal epithelial cell tight junctions and impair the functioning of the gut barrier, leading to bacterial translocation and enhanced entry of bacteria metabolites into the portal vein[36].

Moreover, the impaired motility of the intestines, increased epithelial permeability, and increased release of pro-inflammatory cytokines in the intestines found in cases of liver cirrhosis with portal hypertension may affect the liver[37].

GUT MICROBIOTA AND LIVER DISEASES

There are several reports on the changes in gut microbiota in liver diseases, and the representative data are summarized in Table 3.

Nonalcoholic fatty liver disease

Nonalcoholic fatty liver disease (NAFLD) is a common disease all over the world, following the increasing prevalence of obesity and metabolic syndrome. About 10% of patients with NAFLD are now thought to progress to non-alcoholic steatohepatitis (NASH) with the potential to develop liver cirrhosis and even hepatocellular carcinoma (HCC). Since it is now evident that the gut microbiota play an important role in energy storage and the subsequent development of obesity[38], the role of gut microbiota in the development and progression of NAFLD has become a focus of research.

Indeed, the compositions of microbiota have been shown to differ in obese and lean individuals, with increased *Firmicutes* and decreased *Bacteroidetes* levels in the obese under the intake of the same amount of food[39]. Extensive analyses of gut microbiota in patients with NAFLD have been performed recently,

Table 3 Changes in gut microbiota in liver diseases

Diseases	Change in microbiota	Ref.
NAFLD	*Bacteroidetes* ↓	
	Prevotella ↑	[41]
	Porphyromonas ↑	
Cirrhosis	*Enterobacteriaceae* ↑	
	Streptococcaceae ↑	[45,46]
	Bifidobacteria ↓	
	Lachnospiraceae ↓	
	Bacteroidetes ↓	
	Firmicutes ↓	[47]
	Streptococcus spp. ↑	
	Veillonella spp. ↑	
Alcoholics	*Bacteroidaceae* ↓	[50,51]
	Prevotellaceae ↑	
Alcoholic liver cirrhosis	*Enterobactericaea* ↑	[53]
Cirrhosis with encephalopathy	*Porphyromonadacae* ↑	[55]
	Alcaligenacae ↑	

NAFLD: Nonalcoholic fatty liver disease.

and individuals with NAFLD were found to show a lower percentage of *Bacteroidetes* and higher levels of *Prevotella* and *Porphyromonas* species compared to healthy controls. Moreover, the predisposition to develop to NAFLD was dependent on the expression of Toll-like receptors (TLR) 4 or 9, or tumor necrosis factor-alpha (TNF-α) receptor[40].

The mechanisms underlying the progression from simple fatty liver to NASH are not fully understood, but a study suggested that NASH harbors modified microbiota that produce endogenous ethanol, leading to the development of NASH[39].

A bacteria-mediated mechanism for the progression of NASH was recently proposed by Imajo *et al*[41]. They found that obesity-induced leptin upregulates CD14 *via* STAT3 signaling, resulting in hyper-responsibility to low-dose LPSs, leading to the liver inflammation and fibrosis of NASH.

Liver cirrhosis

In liver cirrhosis, the decreased secretions of bile acid[42,43] and portal hypertension[44] could affect the composition and the growth of gut microbiota. Previous studies[45,46] revealed that the gut microbiota in patients with liver cirrhosis showed a higher prevalence of pathogenic bacteria such as *Enterobacteriaceae* and *Streptococcaceae* and a lower prevalence of beneficial bacteria such as *Bifidobacteria* and *Lachnospiraceae* compared to healthy controls. The gut microbial communities were the same irrespective of their etiologies, indicating that the composition characteristics in the liver cirrhosis patients were due mostly to the liver cirrhosis. Interestingly, a positive correlation was observed between the patients' Child-Turcotte-Pugh (CTP) scores and *Streptococcaceae*, whereas *Lachnospiraceae* was significantly decreased in the cirrhosis patients and negatively correlated with the CTP scores, suggesting a contribution of gut microbiota to the prognosis of patients with liver cirrhosis[45,46].

Qin *et al*[47] analyzed the gut microbiomes in liver cirrhosis patients by quantitative metagenomics, and they found that the gut microbiota in the patients contained less *Bacteroidetes* and *Firmicutes* and more *Streptococcus* spp. and *Veillonella* spp. compared to healthy controls. Both *Streptococcus* spp. and *Veillonella* spp. are bacteria of oral origin and might be associated with the pathophysiology of liver cirrhosis. Although the functions and the contribution of these bacteria in the pathogenesis and complications of liver cirrhosis remain to be clarified, these findings could help develop a new therapeutic strategy against liver cirrhosis by focusing on the gut microbiota.

Alcoholic liver disease

Chronic alcohol consumption is related to fatty liver, liver fibrosis and liver cirrhosis, and both alcohol and acetaldehyde have been suspected to be pathogenic for liver injury. However, the importance of gut microbiota in the pathogenesis of alcoholic liver injury has been revealed. In alcohol-fed mice, *Akkermansia* and *Bacteroides* were increased, and *Lactobacillus* was decreased[48]. In human alcoholics, a decrease in *Bacteroidaceae* and an increase in *Prevotellaceae* were found[49]. Besides gut dysbiosis, increased gut permeability-due to the disruption of tight junctions caused by ethanol and acetaldehyde-leads to the increased entry of LPS, endotoxin and bacterial DNA into the liver[50,51]. These activate Kupffer cells *via* TLR4 or TLR 9 on the cell surface, and induce pro-inflammatory cytokines from Kupffer cells[52].

Tuomisto *et al*[53] analyzed bacterial DNA in the feces of patients with alcoholic liver cirrhosis, and they found that the feces contained more bacterial DNA of *Enterobactericaea* compared to those of healthy volunteers. They also analyzed ascites, and found that 50% of the ascites from the alcoholic liver cirrhosis patients contained *Enterobactericaea*, a *Clostridium leptum* group or *Lactobacillus* spp.[53].

Hepatic encephalopathy

Hepatic encephalopathy (HE) is a complication often found in patients with advanced liver cirrhosis. It greatly affects the quality of life in those patients. HE is characterized by reversible cognitive impairment which is caused not by organic organ damage but by toxic substances produced by microbiota in the intestine. Although ammonia is thought to be the main factor causing HE, other factors such as mercaptans, phenols, short- and medium-chain fatty acids and benzodiazepine-like compounds could contribute to the development of HE. Since most of these factors are derived from gut microbiota, analyses of the composition of the microbes will be important for both the understanding and management of HE.

A report from Bajaj *et al*[54] found that fecal

microbiota in cirrhotic patients contained significantly higher levels of *Enterobacteriaceae*, *Alcaligeneceae*, and *Fusobacteriaceae* and lower levels of *Ruminococcaceae* and *Lachnospiraceae* compared to those in controls. Moreover, in cirrhotic patients with HE, the correlation between *Porphyromonadacae* and *Alcaligenacae* with poor performance on cognitive tests was observed. However, Bajaj *et al*[55] later reported that sigmoid colon mucosal microbiomes are different from stool microbiomes in patients with liver cirrhosis and HE, and they found that in patients with HE the mucosal microbiome, but not the stool microbiome, showed less *Roseburia* and more *Enterococcus*, *Veillonella*, *Megasphaera*, and *Burkholderia* compared to non-HE subjects. These data suggest that a stool microbiota analysis might not be enough to understand the pathogenesis of HE, and that changes in the colonic mucosal microbiota could contribute to the development of HE.

HCC

Eighty percent of HCCs develop in fibrotic or cirrhotic livers, and the main etiologies include hepatitis B, hepatitis C, and alcohol intake. However, as obesity has become more prevalent in most developed countries, the development of HCC in patients with NAFLD has been increasing. In viral hepatitis, chronic inflammation of the liver is thought to be associated with hepatocarcinogenesis, but the mechanism underlying the development of HCC in NAFLD patients has not been clarified.

Yoshimoto *et al*[56] reported that obese mice show alterations of gut microbiota, leading to an increased production of a gut bacterial metabolite, deoxycholic acid (DCA), which is known to cause DNA damage. Increased levels of DCA in the enterohepatic circulation induce a senescence-associated secretory phenotype in the hepatic stellate cells, which secrete inflammatory and tumor-promoting factors in the liver. The obese mice showed HCC development after exposure to a chemical carcinogen. These data indicate that gut bacterial metabolites promote obesity-induced HCC development in mice. Yoshimoto *et al*[56] also found that a similar pathway may contribute to NASH-associated HCC development in humans.

Dapito *et al*[57] demonstrated in a mouse model that TLR4 activation by LPS from the intestinal microbiota was closely associated with injury- and inflammation-driven tumor promotion but not with the tumor initiation of hepatocarcinogenesis induced by a combination of diethylnitrosamine and the hepatotoxin carbon tetrachloride. The contribution of intestinal microbiota was confirmed by a decrease in hepatocarcinogenesis in germ-free mice compared to specific pathogen-free mice. On the other hand, a metabolite from gut microbiota, propionate, was shown to inhibit the cancer cell proliferation of liver cancer cells in the liver in a mouse model[58].

Intratumoral interleukin-17-producing T helper cells (Th17) were found to be associated with poor prognoses of patients with HCC[59], possibly due to the promotion of angiogenesis and tumor growth[60], and most of these Th17 cells are generated in the gut by an interaction with gut microbiota[61]. Therefore, there could be close associations among HCC progression, the generation of Th17 cells, and gut microbiota. Taken together, the above-described findings indicate that the development of liver cancer can be modulated by gut microbiota, suggesting the possibility of therapeutic intervention.

UTILIZATION OF PROBIOTICS IN LIVER DISEASES

Bacterial translocation and endotoxemia, which can be caused by increased permeability of the intestinal mucosa[62], are thought to contribute to the pathogenesis of various complications in liver cirrhosis such as hepatic encephalopathy[63,64] and spontaneous bacterial peritonitis[65,66]. The changes of intestinal microflora might also be associated with the pathogenesis of NASH[67]. Beneficial effects of probiotics have been reported in not only gastrointestinal but also liver diseases. Here we describe therapeutic trials using probiotics and their indications in liver diseases.

HE

HE is a common complication of liver cirrhosis[63]. Gut flora are associated with the pathogenesis of HE[64], because urease-producing gut bacteria such as *Klebsiella* and *Proteus* species can increase the production of ammonia and endotoxins. It was reported in several studies that the alteration of gut flora using probiotics and/or prebiotics could improve HE. The probiotics include strains of lactic acid bacilli (*e.g.*, *Lactobacillus* and *Bifidobacterium*), a nonpathogenic strain of *Escherichia coli* (*e.g.*, *E. coli* Nissle 1917), *Clostridium butyricum*, *Streptococcus salivarius*, and *Saccharomyces boulardii* (a nonpathogenic strain of yeast), and VSL#3. The most-studied probiotic, VSL#3, consists of a mixture of eight probiotic strains (*Streptococcus thermophilus, Bifidobacterium breve, B. longum, B. infantis, Lactobacillus acidophilus, L. plantarum, L. paracasei,* and *L. bulgaricus*)[68].

As of 2011, nine randomized controlled trials (RCTs) had been reported comparing probiotics and synbiotics with no intervention or placebo in patients with HE (Table 4)[69-77]. Some of these studies included cases using lactulose for prebiotics or as control arms; lactulose acts by altering the colonic pH and improving gastrointestinal transit. McGee *et al*[78] and Holte *et al*[79] independently reported a systematic review and a meta-analysis of these randomized trials on probiotics for HE, respectively. Both groups found that patients treated with probiotics appeared to have reduced plasma ammonia concentrations compared

Table 4 Randomized controlled trials for hepatic encephalopathy

Ref.	Year	Sample size (treatment/placebo)	Treatment regimens	Duration	Favorable effects
[1,2]Loguercio *et al*[69]	1987	40 (20/20)	*Enterococcus Lactic Acid bacteria* strain SF68 *vs* lactulose	10 d	NH_3 ↓ Performance status: improved
[2]Loguercio *et al*[70]	1995	40 (21/19)	*Enterococcus Lactic Acid bacteria* strain SF68 *vs* lactulose	3 x 4 wk	NH_3 ↓ Psychometric test: improved
[1,2]Liu *et al*[71]	2004	55 (20/35)	*Pediacoccus pentoseceus, Leuconostoc mesenteroides, Lactobacillus paracasei*, and *Lactobacillus plantarum* with fermentable fibers *vs* fermentable fibers only or non-fermentable fiber	30 d	Endotoxemia ↓ Child-Turcotte-Pugh classification: improved
[2]Malaguarnera *et al*[72]	2007	60 (30/30)	*Bifidobacterium* (subtype not available) with fructo-oligosaccharide (FOS) *vs* mix of vitamins	90 d	NH_3 ↓ Psychometric test: improved
[1,2]Bajaj *et al*[73]	2008	25 (17/8)	*Streptococcus thermophilus, Lactobacillus bulgaricus, Lactobacillus acidophilus, Lactobacillus casei*, and *Bifidobacteria vs* none	60 d	Psychometric test: improved
[1,2]Sharma *et al*[74]	2008	105 (70/35)	*Streptococcus faecalis, Clostridium butyricum, Bacillus mesentricus*, and *Lactic acid bacillus* with lactulose *vs* lactulose	30 d	NH_3 ↓ Psychometric test: improved
[1,2]Mittal *et al*[75]	2011 (2009)	160 (120/40)	VSL#3 (containing *Streptococcus thermophilus, Bifidobacterium breve, Bifidobacterium longum, Bifidobacterium infantis, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus paracasei, Lactobacillus bulgaricus*) *vs* lactulose or placebo	3 mo	NH_3 (arterial) ↓
[1]Malaguarnera *et al*[76]	2010	125 (63/62)	*Bifidobacterium* (subtype not available) and FOS *vs* lactulose	60 d	NH_3 ↓ Psychometric test: improved
[1]Pereg *et al*[77]	2011	40 (20/20)	*Lactobacillus acidophilus, Lactobacillus bulgaricus, Bifidobacterium bifidum*, and *Streptococcus thermophiles* (Bio-plus, Supherb, Israel) *vs* placebo	6 mo	NH_3 ↓
Agrawal *et al*[80]	2012	235 (157/78)	VSL#3 *vs* lactulose or none	> 3 mo	NH_3 (arterial) ↓ Psychometric test: improved Secondary prophylaxis of HE
Lunia *et al*[81]	2014	160 (86/74)	VSL#3 *vs* placebo	> 6 mo	NH_3 (arterial) ↓ Prevention of HE

[1]RCTs included in meta-analysis by McGee *et al*[78]; [2]RCTs included in meta-analysis by Holte *et al*[79].

to patients treated with placebo or no intervention, but treatment with neither probiotics nor synbiotics did not significantly alter the clinically relevant outcomes (*i.e.,* mortality and quality of life). The trials had high risks of systematic and random errors, because each sample size was small and the dosing periods and quantities of the probiotics were different among the trials.

After those meta-analyses, Agrawal *et al*[80] reported a randomized trial examining the use of probiotics for the secondary prophylaxis of HE. The study assigned 235 patients who had suffered from HE previously, and the results showed that recurrent HE occurred in fewer patients who received probiotics or lactulose compared with no treatment (34% and 27%, respectively, *vs* 57%). There was no significant difference in recurrence rates between the patients who received probiotics and those who received lactulose. Lunia *et al*[81] reported preventive effects of probiotics on the development of HE in patients with liver cirrhosis who had not experienced overt HE. Patients who received the probiotics were less likely to develop overt HE compared to controls (1.2% *vs* 19%; HR = 2.1). These prospective trials indicate that probiotics could be effective in preventing overt HE.

On the other hand, *Lactobacillus* GG AT strain 53103 (LGG), which is a well-studied probiotic with a published history of safety and efficacy in humans, was examined to evaluate its safety and tolerability in patients with minimal HE[82]. The trial showed that LGG was safe and well-tolerated in patients with cirrhosis and could cause reductions of the endotoxemia and TNF-α production seen in these patients[82].

Among the various probiotics, the most efficacious species for HE appeared to be *Lactobacilli* and *Bifidobacteria*. However, additional prospective and randomized controlled trials are needed before these probiotics can be routinely recommended.

Non-alcoholic steatohepatitis

NAFLD is characterized by the accumulation of triglyceride in hepatocytes in non-alcohol users. NAFLD can progress to more severe liver diseases, such as NASH[83,84]. It has been reported the NAFLD/NASH was associated with increased intestinal permeability and small bowel bacterial overgrowth, which could increase the production of proinflammatory cytokines

Table 5 Randomized controlled trials for non-alcoholic steatohepatitis

Ref.	Year	Sample size (treatment/placebo)	Treatment regimens	Duration	Favorable effects	Other information
[1]Aller *et al*[90]	2011	28 (14/14)	*Lactobacillus bulgaricus* and *Streptococcus thermophiles vs* placebo	3 mo	ALT ↓	Cardiovascular risk factors: NS
[1]Vajro *et al*[91]	2011	20 (10/10)	*Lactobacillus rhamnosus strain GG vs* placebo	8 wk	ALT ↓ PG-PS IgA ↓	Hepatorenal US ratio: NS
[1]Malaguarnera *et al*[92]	2012	66 (34/32)	*Bifidobacterium longum* with *fructo-oligosaccharides vs* placebo	24 wk	ALT ↓ CRP ↓ TNF-α ↓ LDL-cholesterol ↓ Serum endotoxin ↓ HOMA-IR↓ Steatosis ↓ NASH activity index ↓	
[1]Wong *et al*[93]	2013	20 (10/10)	*Lactobacillus plantarum, Lactobacillus deslbrueckii, Lactobacillus acidophilus, Lactobacillus rhamnosus* and *Bifidobacterium bifidum* (The Lepicol probiotic formula) *vs* usual care	6 mo	AST ↓ Liver fat (IHTG) ↓	

[1]RCTs included in meta-analysis by Ma *et al*[94]. PG-PS: Peptidoglycan-polysaccharide; IHTG: Intrahepatic triglyceride content; NASH: Non-alcoholic steatohepatitis; NS: Not significant.

and contribute to the pathogenesis of NASH[85]. Several pharmacologic treatments for NAFLD/NASH have been reported, including insulin-sensitizers (*e.g.*, pioglitazone), antioxidants (*e.g.*, N-acetylcysteine, vitamin E), ursodeoxycholic acid, and anti-TNF-α agents, but a standard treatment has not been established[86].

In animal models of NASH, treatments with probiotics such as VSL#3 improved the histological findings including reduced fat deposits and damage to the liver parenchyma, with decreasing serum alanine aminotransferase (ALT) levels[87,88]. VSL#3 also reduced oxidative and inflammatory liver damage in mouse NASH models[89].

Several clinical trials of probiotics administered to patients with NAFLD have been reported. Loguercio *et al*[70] found that the administration of VSL#3 might reduce liver injury and improve liver function in NAFLD patients. Four relatively high-quality RCTs that evaluated the effects of probiotics in NAFLD patients were reported (Table 5)[90-93]. All four trials showed that probiotic therapy significantly decreased the serum levels of ALT, but only two of the trials revealed an improvement of liver steatosis, which was evaluated by repeated liver biopsies[92] or proton-magnetic resonance[93]. Ma *et al*[94] performed a meta-analysis of these four RCTs to assess the efficacy of probiotic therapies, and they found that the probiotic therapy significantly decreased the levels of aminotransferases, total-cholesterol, high-density lipoprotein (HDL) and TNF-α in the serum, and the homeostasis model assessment of insulin resistance. The lower levels of HDL in the probiotic-treated groups compared to the placebo groups was unexpected, and the mechanism is unclear. In two of the RCTs, probiotics were used with prebiotics in NAFLD patients, and the combination significantly reduced the patients' serum aminotransferase levels and liver steatosis.

Lactobacillus, *bifidobacterium*, and *streptococcus* are used as probiotics for patients with NASH, and prebiotics such as fructo-oligosaccharides (FOS) are frequently used with these probiotics because they can be fermented by *bifidobacteria* and *lactobacilli*[95]. FOS could contribute to *bifidobacteria* growth as the dominant species in the large bowel and reduce the growth of harmful bacteria[96].

Although the above-described studies have several limitations including small sample sizes and a lack of data about the patients' diets and exercise, the treatments with probiotics and prebiotics for patients with NAFLD are promising.

Alcoholic liver disease

The chronic consumption of alcohol induces various biological abnormalities including liver injury (ranging from fatty liver, liver fibrosis, and alcoholic hepatitis to liver cirrhosis), pancreatitis, impaired neutrophil functions, endotoxemia, and increased oxidative stress in the gut. Most of these abnormalities could be associated with altered gut microbiota, raising the possibility that probiotics would be effective for the improvement of these conditions.

In an open-label study with *Lactobacillus casei Shirota*[97], patients with alcoholic cirrhosis who received the probiotic for 4 wk showed a restoration of neutrophil phagocytic capacity, possibly due to the decreased expression of TLR4 on the surface of these cells. In a mouse model fed an ethanol-containing diet, heat-killed *Lactobacillus brevis* (*L. brevis*) SBC8803 significantly decreased the serum levels of ALT and AST and the hepatic content of triglyceride and total cholesterol in the liver caused by ethanol, which may be associated with the up-regulation of TNF-α and sterol regulatory element-binding proteins in the liver[98].

In a pilot study reported by Kirpich *et al*[99], alcoholic patients who received *bifidobacteria* and

lactobacilli for 5 d showed increased numbers of those bacteria in the gut, and they showed significantly lower AST and ALT activity in the serum, indicating that a short-term administration of probiotics can change the gut microbiota, leading to the amelioration of liver injury induced by chronic alcohol consumption. The therapeutic effect of probiotics could be due to a reduction in oxidative stress and inflammation in the intestine and liver and the preservation of gut barrier function[100]. *L. plantarum* was recently microencapsulated and administered to rats chronically fed alcohol[101]. The rats showed reductions in the levels of endotoxemia, serum aminotransferase, NF-κB, cytokines such as TNF-α, and IL12/p40, and they also showed improvements in the histology of the intestine and liver. Considering the greater survival of probiotics under gastric acidic conditions, this approach might be efficacious. However, the bacteria-free culture supernatant of *Lactobacillus rhamnosus GG* was proven to suppress the alcohol-induced intestinal permeability, endotoxemia and subsequent liver injury[102]. It thus remains uncertain whether the survival of probiotics is necessary for obtaining the optimal effect of treatment with probiotics.

HCC

Most HCCs develop in the liver chronically infected by Hepatitis B or C virus. However, with the increasing prevalence of metabolic syndrome, the incidence of HCC from NASH has been increasing[103]. Although the mechanism of HCC development from NASH in association with dysbiosis of gut microbiota has been proposed as mentioned above, no therapeutic trials for the prevention of HCC in patients with NASH have been reported, to our knowledge. Reports of the therapeutic prevention of HCC using probiotics are limited to aflatoxin-induced HCC. In a clinical study, healthy but aflatoxin-exposed subjects were randomly assigned to two groups; one group received a mixture of *Lactobacillus rhamnosus LC705* and *Propionibacterium freudenreichii* subsp. *shermanii* strains and the other received a placebo, and the groups' urinary excretions of aflatoxin-DNA adduct (aflatoxin B(1)-N(7)-guanine) were compared[104]. The results showed that the elevated urinary excretion of aflatoxin B(1)-N(7)-guanine was significantly decreased in the probiotics group but not in the controls, suggesting the inhibition of the intestinal absorption of aflatoxin B(1) by probiotics[104]. These data may also indicate that probiotics therapy could contribute to the inhibition of aflatoxin B-induced hepatocarcinogenesis. In a rat study examining the effects of probiotic fermented milk and chlorophyllin on the prevention of aflatoxin-induced hepatocarcinogenesis, the probiotics treatment reduced the expressions of c-myc, bcl-2, cyclin D1 and rasp-21, suggesting the protective potential of the probiotics in aflatoxin-induced hepatocarcinogenesis[105].

Surgical procedures

Probiotic and symbiotic therapies have been attempted to maintain liver function or prevent post-surgical infections in patients undergoing liver resection or liver transplantation.

Rayes reported a randomized double-blind study with a composition of four lactic acid bacteria *(Pediacoccus pentosaceus* 5-33:3, *Leuconostoc mesenteroides* 77:1, *Lactobacillus paracasei* ssp. *paracasei* F19, and *L. plantarum* 2362) and four fibers[106]. The treated group received probiotics for 15 d, and the control group received fibers only. The treated group showed less post-operative infections (3% in the treated group *vs* 48% in the control group) and needed shorter antibiotic therapy compared to the control group. Similar results were obtained in a study conducted in Japan[107] in which probiotics treatment reduced the perioperative infections in liver transplantation recipients from 24% to 4%. In another study, treatment with probiotics 3 d preoperatively and 7 d postoperatively was found to be efficacious for the better and faster recovery of liver functions[108]. These data suggest that perioperative probiotics or synbiotics may have significant benefits by reducing infections and by maintaining liver functions in patients undergoing liver resection or liver transplantation.

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Robotic assisted Roux-en-Y hepaticojejunostomy in a post-cholecystectomy type E2 bile duct injury

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Abstract

Roux-en-Y hepaticojejunostomy anastomosis is the treatment of choice for common hepatic duct injury type E2. It has been performed laparoscopically with the advancement of laparoscopic skill. Recently, a telemanipulative robotic surgical system was introduced, providing laparoscopic instruments with wrist-arm technology and 3-dimensional visualization of the operative field. We present a case of 36-year-old female patient who had undergone elective cholecystectomy 2 mo ago for gall stones and had a common bile duct injury during surgery. As the stricture was old and complete it could not be tackled endoscopically. We did a laparoscopic assisted adhesiolysis followed by robotic Roux-en-Y hepaticojejunostomy. No intraoperative complications or technical problems were encountered. Postoperative period was uneventful and she was discharged on the 4th postoperative day. At follow-up, she is doing well without evidence of jaundice or cholangitis. This is the first reported case of robotic hepaticojejunostomy following common bile duct injury. The hybrid technique gives the patient benefit of laparoscopic adhesiolysis and robotic suturing.

Key words: Hepaticojejunostomy; Common bile duct; Robotic surgery; Cholecystectomy complication; Hepatic duct

Core tip: Robotic surgery has the advantages of a 20 times magnified 3D vision for the surgeon. The intuitive and tremor free accurate movements of the robotic fingers make it an ideal tool to do fine reconstructive surgery. Hepaticojejunostomy is one such surgery specially when done for bile duct injury that occurred during cholecystectomy. We present the first ever reported robotic hepaticojejunostomy for iatrogenic bile duct injury.

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INTRODUCTION

Although seen with any operation involving right upper quadrant dissection, injuries occurring during or following cholecystectomy account for more than 80% of all iatrogenic bile duct injuries. During laparoscopic or open cholecystectomy, injury to the common bile duct is an unusual but devastating complication[1]. Inflammation in the porta, variable biliary anatomy, inappropriate exposure, aggressive attempts at hemostasis, and surgeon inexperience are commonly cited risk factors. Early reports suggested that surgical inexperience, performing less than 20 laparoscopic cholecystectomies, was highly correlated with bile duct injury, evidence has suggested that visual misperception accounts for 97% of iatrogenic biliary injuries and technical skill or knowledge accounts for only 3%. With sufficient cephalad retraction of the gallbladder fundus, the cystic duct overlies the common hepatic duct, running in a parallel path[2]. Without inferolateral traction of the gallbladder infundibulum to dissociate these structures, dissection of the apparent cystic duct may actually include the common hepatic duct, placing it in jeopardy. By retracting Hartmann's pouch inferolaterally, and opening the triangle of Calot, the cystic duct is displaced from the porta, no longer collinear with the hepatic duct. The use of a 30-degree laparoscope is useful to provide adequate visualization of the critical view of safety during laparoscopic cholecystectomy[3]. Also, in many of these cases, a confirmation bias occurs, in which surgeons tend to rely on evidence that supports their perception while simultaneously discounting visual cues that suggest an alternative explanation. Confirmation bias helps explain why most bile duct injuries are identified in the postoperative setting, not intraoperatively. The multifactorial nature of biliary injury highlights the concept that injury avoidance consists of many levels of protective mechanisms[4]. Surgeon knowledge of biliary anatomy and aberrant anatomy, use of an angled laparoscope, appropriate and directed traction and counter-traction on the gallbladder, sufficient suspicion of findings that discount the current perspective, and converting to an open operation at the earliest suspicion can help decrease the likelihood of biliary injury. Although the use of routine versus selective cholangiography is controversial, evidence has suggested that cholangiography does not completely avoid bile duct injury, but may reduce the incidence and extent of injury[5]. The original analysis of biliary reconstruction was based on the Bismuth classification, and has been modified by Strasberg. Classification of bile duct injuries is determined by location of injury and helps guide later surgical reconstruction[6]. Among postoperative bile duct strictures, types E1 and E2 involve the common hepatic duct but not the bifurcation, with type E1 having a stump of more than 2 cm of the common hepatic duct below the confluence and type E2 being within 2 cm of the confluence. Type E3 strictures occur at the confluence preserving the extrahepatic ducts and, in type E4, the structuring process includes the extrahepatic biliary tree. Type E5 strictures involve aberrant right hepatic duct anatomy, with injury to the aberrant duct and common hepatic duct.

The standard treatment procedure for a type E2 stricture is a resection of the injured segment with mucosa to mucosa anastomosis using a Roux-en-Y jejunal limb[7]. Choledochoduodenostomy is not done because most injuries to the bile duct occur higher in the biliary tree, close to the hilum, thus not allowing for tension-free anastomosis to the duodenum. Recently, many centers have reported their experience with laparoscopic Roux-en-Y hepaticojejunostomy. Although the use of laparoscopy to do the procedure has found to be feasible, most of these reports suggested that these procedures were complicated by technical difficulties. The use of da Vinci Robotic Surgical System helps decrease these technical difficulties. The da Vinci robot provides a three-dimensional vision instead of a two dimensional one through the laparoscope. The Robotic system also provides other advantages like reduction of tremor and scaling of motion which further increase the precision of the procedure. But the biggest advantage is the use wristed instrumentation which provides additional degrees of mobility compared to the restriction of the laparoscopic systems. We used the daVinci robot to do an Roux-en-Y Hepaticojejunostomy in a 36 years old female patient with Type E2 CBD injury post cholecystectomy.

CASE PRESENTATION

Our patient was a female of 36 years and came to us with multiple episodes of abdominal pain with deep icterus. She gave history of undergoing an elective open cholecystectomy at another hospital two months ago for gall stone disease. Her liver function tests were heavily deranged with high direct bilirubin (> 20 mg/dL) and OT/PT (538/422 mg/dL) and alkaline phosphatase (758 mg/dL). Ultrasonography demonstrated mild hepatomegaly with dilated intrahepatic biliary radicles (Figure 1). An magnetic resonance cholangiopancreatography scan (Figure 2) revealed a tight short segment stricture in the common hepatic duct with gross intrahepatic biliary radical dilatation. Opinion was sought from gastroenterologist who felt that the stricture is not passable by Encoscopic Retrograde Cholangio-Pancreatography and surgical option has to be tried. The patient underwent laparoscopic adhesiolysis and da Vinci robotic Roux En Y hepaticojejunostomy.

SURGICAL TECHNIQUE

After placing the patient in supine position, pneumoperitoneum was created upto 12 mmHg using the Veress needle. Three 5 mm trocars were inserted to

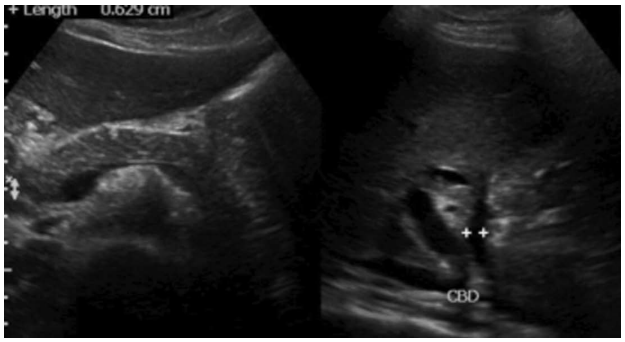

Figure 1 Ultrasound showing dilated hepatic ducts.

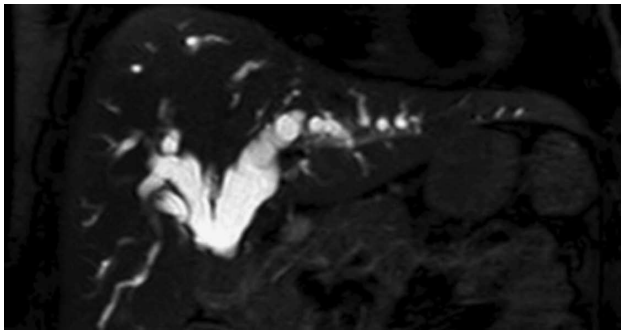

Figure 2 Mri scan of the hepatic ducts.

carry out the adhesiolysis. We then placed the camera and instruments and the table was placed in reverse Trendelenburg and right up position. This allowed the intestines to fall away from the operating field Alongwith cephalad retraction to the liver the porta hepatis was exposed. Laparoscopic adhesiolysis was done with the help of a suction and cautery. The porta was freed from the duodenum and the transverse colon. Bile was aspirated from the confluence.

The robot was docked. Three 12-mm robotic trocars for camera and accessory device were applied. The jejunum was divided at 40 cm from the DJ flexure by an endoscopic stapler. Side-to-side enteroenterostomy anastomosis was done with the help of endoscopic stapler. The bile duct was opened (Figure 3) and was anastomosed to a Roux loop of jejunum with an antecolic Roux-en-Y end-to-side hepaticojejunostomy. The anastomoses was made using 3 0 Vicryl sutures at each of the corners (Figure 4). The hepatico-jejunostomy and jejuno-jejunostomy were re-checked for proper lie, bile leak and hemostasis. A drain was placed in the subhepatic region. The fascial and skin incisions were closed.

POSTOPERATIVE COURSE

Postoperatively, the patient was managed conservatively with i.v. antibiotics, analgesics and fluids. The subhepatic drain showed minimal drainage and was removed on second post operative day. She was started on liquids on the third post operative day and progressed to normal diet on the fourth postoperative day. She was discharged without any complications. On follow up, she is doing well without any complaints.

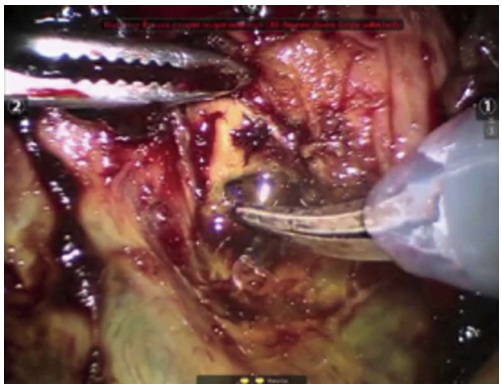

Figure 3 Hepatic duct opened.

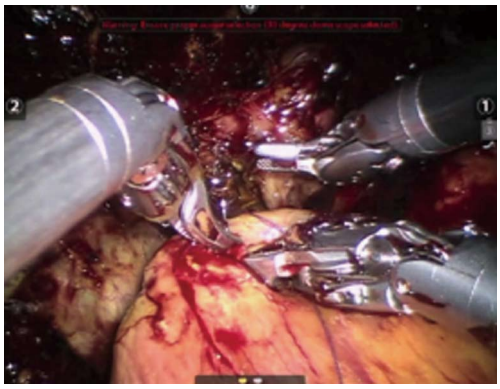

Figure 4 Robotic hepaticojejunostomy anastomosis.

DISCUSSION

The first uses of laparoscopic surgery were in the field of hepato-biliary surgery *i.e.*, cholecystectomy. Following advancements in the camera and instruments and the increased skill of surgeons, Minimal access surgery has invaded all parts of the body today. But the use of laparoscopy in the field of hepato-biliary surgery still remains restricted to cholecystectomies. This is mostly due to the limited exposure present in the area, along with dense adhesions increasing the risk of visceral injury. The instruments of laparoscopy are rigid in nature with limited degrees of freedom. This coupled with the fulcrum effect of laparoscopy which takes the movement away from the wrist and fingers to the elbow and 2-dimensional imaging, are the main contributors to the limitations of the laparoscopic approach[8]. Robotic surgery becomes a great tool in hepato biliary by overcoming these shortcomings.

The daVinci robot has a three dimensional vision compared to the two dimensional view of the laparoscope which provides for the surgeon to be immersed in the oppressive field. Secondly, the motion scaling

and tremor reduction software increase the precision of the surgery and decrease blood loss. However, the main advantage of the da Vinci robot is the use of the wristed instruments for doing fine dissection in areas of dense adhesions and fine suturing of hepaticojejunostomy and jejunojejunostomy anastomoses[9]. It might well be that in the future, advanced hepato biliary procedures like hepaticojejunostomy and Whipple's procedure may be done by robotic surgery.

Robotic surgery, with its implications in urologic and gynaecologic surgery, has had multiple mention in case reports. A few cases of hepaticojejunostomy for choledochal cysts have also been reported but no reports have been found for a Roux-en-Y hepaticojejunostomy for a biliary injury following cholecystectomy[10,11].

On the other side of the coin, the disadvantages includes the bulky hardware which makes it impossible for the robot to be moved to other theatres. Although the lack of haptic feedback and loss of tactile sensation are propagated by noon believers to be the biggest obstacle, but the magnified three dimensional vision more than compensates for it. Finally, in a country like India, the increased initial cost of the machine along with high maintenance cost prevents it's penetration into peripheral areas[12]. Although many people advocate a steep learning curve for advanced laparoscopic surgery, but the ease of dissection and suturing techniques with the robotic technique greatly reduce the learning curve.

Finally, we would like to say that the use of robotic surgery has immense scope in hepato biliary surgery with advanced surgeries like hepaticojejunostomy, CBD exploration and pancreaticoduodenectomy being facilitated by the ease of Robotic suturing[13].

CONCLUSION

Summarising we are reporting a case of 36 years old female with a type E2 biliary injury and the use of daVinci surgical system to create a Roux-en-Y Hepaticojejunostomy. The most technically challenging step of this operation is the creation of the hepaticojejunostomy anastomosis. The daVinci surgical system triumphs over laparoscopy due to the ease of the suturing with the endowristed instruments. This is the first reported case of robotic Roux-en-Y hepaticojejunostomy following common bile duct injury. The hybrid technique gives the patient benefit of laparoscopic adhesiolysis and robotic suturing. But further experience is needed to evaluate the advantages on surgical technique and it is effect on decreased hospital stay for patients.

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REVIEW

Pancreatic cancer early detection: Expanding higher-risk group with clinical and metabolomics parameters

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is the fourth and fifth leading cause of cancer death for each gender in developed countries. With lack of effective treatment and screening scheme available for the general population, the mortality rate is expected to increase over the next several decades in contrast to the other major malignancies such as lung, breast, prostate and colorectal cancers. Endoscopic ultrasound, with its highest level of detection capacity of smaller pancreatic lesions, is the commonly employed and preferred clinical imaging-based PDAC detection method. Various molecular biomarkers have been investigated for characterization of the disease, but none are shown to be useful or validated for clinical utilization for early detection. As seen from studies of a small subset of familial or genetically high-risk PDAC groups, the higher yield and utility of imaging-based screening methods are demonstrated for these groups. Multiple recent studies on the unique cancer metabolism including PDAC, demonstrate the potential for utility of the metabolites as the discriminant markers for this disease. In order to generate an early PDAC detection screening strategy available for a wider population, we propose to expand the population of higher risk PDAC group with combination clinical and metabolomics parameters.

Key words: Pancreatic cancer; Endoscopic ultrasound; Metabolomics; Early detection; Biomarkers

Core tip: This is a summary of current pancreatic cancer cohort early detection studies and a potential approach being considered for future application. This is an area that requires heightened efforts as lack of effective treatment and screening scheme for wider population is leading this particular disease to be the second lethal cancer by 2030.

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INTRODUCTION

Currently, pancreatic ductal adenocarcinoma (PDAC) is the fourth major cause of cancer mortality in the United States[1]. It is predicted that 46420 new cases and 39590 deaths would result from pancreatic cancer in the United States in 2014[2]. Worldwide, there were 277668 new cases and 266029 deaths from this cancer in 2008[3]. In comparison to other major malignancies such as breast, colon, lung and prostate cancers with their respective 89%, 64%, 16%, 99% 5-year survival rate, PDAC at 6% is conspicuously low[2]. For PDAC, the only curative option is surgical resection, which is applicable in only 10%-15% of patients due to the common discovery of late stage at diagnosis[4]. In fact, PDAC is notorious for late stage discovery as evidenced by the low percentage of localized disease at diagnosis, compared to other malignancies: breast (61%), colon (40%), lung (16%), ovarian (19%), prostate (91%), and pancreatic cancer (7%)[5]. With the existing effective screening methods, the decreasing trends of cancer death rate are seen in major malignancies such as breast, prostate and colorectal cancer. In contrast, it is estimated that PDAC is expected to be surfacing as the second leading cause of cancer death by 2030[6].

With the distinct contribution of late-stage discovery and general lack of effective medical therapy, a critical approach in reversing the poor outcome of pancreatic cancer is to develop an early detection scheme for the tumor. In support of this, we see the trend that despite the poor prognosis of the disease, for those who have undergone curative resection with negative margins, the 5-year survival rate is 22% in contrast to 2% for the advanced-stage with distant metastasis[7,8]. An earlier diagnosis with tumor less than 2 cm (T1) is associated with a better 5-year survival of 58% compared to 17% for stage ⅡB PDAC[9]. Ariyama *et al*[10] reported complete survival of 79 patients with less than 1 cm tumors after surgical resection. Furthermore, as a recent report indicates, the estimated time from the transformation to pre-metastatic growths of pancreatic cancer is approximately 15 years[11]; there is a wide potential window of opportunity to apply developing technologies in early detection of this cancer.

In this article, we will review the recent studies on the PDAC early detection approaches and ongoing research endeavors in developing early detection schemes for this devastating disease, with specific attention to application of combined clinical and metabolomics parameters.

PDAC EARLY DETECTION AND DIAGNOSIS - IMAGING-BASED TESTS

Over the past few decades, endoscopic ultrasound (EUS) has proven itself to be a superior imaging study for detection of a small or early-stage pancreatic neoplasm as compared to other modalities such as transabdominal ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography scans and angiography[12-14]. Yasuda *et al*[15] and Rösch *et al*[16] had initially demonstrated the superiority of EUS in detection of small pancreatic lesions. More recently, DeWitt *et al*[17] had verified the superiority of EUS as compared to multi-detector CT scan. In another study, Khashab *et al*[18] demonstrated that the sensitivity of EUS in detecting a pancreatic mass was significantly greater than that of CT images, and particularly for pancreatic neuroendocrine tumors, which commonly consist of smaller lesions. In addition, EUS detected CT-negative tumors in more than 90% of the cases. As an additional diagnostic modality, EUS-guided fine needle aspiration (FNA) provides success rates of 90%-95%, with an overall sensitivity and specificity of 85%-90% and 98%-99%, respectively[19-22]. Thus, the utility and the advantage of EUS enable visualization and targeting of small pancreatic masses. Lesions of 5 mm or less could be visualized and sampled, which might not have been accessible or identifiable by other imaging modalities[23].

DIAGNOSTIC MOLECULAR MARKERS AND PANCREATIC CANCER

In order to enhance the diagnostic accuracy of PDAC, molecular markers on EUS-FNA samples have been evaluated in recent years. Utilities of DNA mutations and loss of heterozygosity are being reported as potential surrogate markers of the cancer[24,25]. In a recent study, Takahashi *et al*[26] assessed *k-ras* point mutations in PDAC and chronic focal pancreatitis samples obtained by EUS-FNA[27,28]. The study revealed the presence of point mutations of *k-ras* in 74% of patients with PDAC compared to no mutations in chronic focal pancreatitis. In another study, Tada *et al*[29] reported a high *k-ras* gene mutation rate in 20 of 26 cases of EUS-FNA specimens (77%) and in 12 of 19 cases of pancreatic juice (63%) in PDAC. However, the presence of *k-ras* mutations in a benign condition such as chronic pancreatitis and premalignant lesions such as intraductal papillary mucinous neoplasm (IPMN) in addition to lack of such mutations in 20% of PDAC limit the usage of this test solely as a diagnostic or a detection tool. Other studies analyzing p53 by immunohistochemistry[30], telomerase activity with a ribonucleoprotein enzyme[31], and a broad panel of microsatellite allele loss markers demonstrated similar results[32]. In the presence of inconclusive EUS-FNA cytology, molecular markers could potentially complement EUS-FNA cytology results to help establish the diagnosis of malignancy.

Table 1 Pancreatic ductal adenocarcinoma related genetic syndromes

Syndrome	Inheritance	Gene mutation	Risk of PDAC
Peutz-Jeghers syndrome[38]	AD	*STK11/LKB1*	SIR = 132
Hereditary pancreatitis[39-41]	AD	*PRSS1*	OR = 69.9
		SPINK1	
Familial atypical multiple mole melanoma syndrome[42-44]	AD	*CDKN2A*	SIR = 13-38
Hereditary breast-ovarian cancer syndrome[45-51]	AD	*BRCA2*	BRCA2: OR = 3.5-10-fold increased risk
		BRCA1	BRCA1: OR = 2.26 times average population
Lynch syndrome[52]	AD	*MLH1, MSH2, MSH6* or *PMS2*	SIR = up to 8.6
Cystic fibrosis[53]	Autosomal recessive	*CFTR*	OR = 5.3-6.6

AD: Autosomal dominant; PDAC: Pancreatic ductal adenocarcinoma; SIR: Standardized incidence ratio.

SELECT POPULATION-BASED RESEARCH FOR EARLY DETECTION SCHEME DEVELOPMENT

PDAC screening in high-risk individuals

Currently, a general population-screening program for PDAC is not cost-effective because of low relative disease incidence and non-availability of simple, cheap, highly accurate non-invasive tests. The main goal of the screening is to identify clinically significant precursor or early stage PDAC. However, since overwhelming majority of premalignant and small PDAC lesions is asymptomatic, we do not have a definite surrogate marker to identify a subset population for screening. Consequently, as one of the approaches in investigating the risks, research has focused on identification of a subset of individuals with a higher-risk for PDAC development in order to elucidate the genetic predilection. Up to 10% of PDAC patients have a familial/genetic basis and they have increased risk of developing both pancreatic and extra-pancreatic malignancies[33-37]. Classic categorization of high-risk patients are based on the highly associated genetic risks defined as those who have significant family history of the cancer or have an inherited PDAC syndrome with a known genetic abnormality (Table 1).

Familial pancreatic cancer: Familial pancreatic cancer (FPC) cohort (cancer in two or more first-degree relatives (FDRs) or in three or more affected family members - including one first-degree relative) is considered a high-risk and a candidate for screening program[47,54,55]. Currently, the genetic foundation for FPC is not fully understood. Various investigations have demonstrated the presence of a germline mutation in the *BRCA2* gene[47-49], association of BRCA1[46,56], *paladin* gene mutation[57] as well as other genes such as apolipoprotein A4, CEA, keratin 19, stratifin, trefoil factor, and S100A6[58,59] in FPC, and more recently identification of PALB2[60], as a pancreatic cancer susceptibility gene. These facts suggest that multiple and heterogeneous factors are likely at play for the genesis of PDAC in this subset.

Analysis of the PDAC kindred data from Johns Hopkins' National Familial Pancreas Tumor Registry has shown that the risk of PDAC in individuals with two afflicted FDRs is 6.4% and the lifetime risk is 8%-12%; for persons with three afflicted FDRs, the relative and lifetime risks for PDAC increase to 32% and 16%-32%, respectively[36]. Brune *et al*[61] in their recent article reported a higher risk of PDAC among FPC kindred with a younger onset (age less than 50). Rulyak *et al*[62] in another study found smoking as a significant risk factor in FPC cohort, especially among males and those under age 50. This factor increases the cancer risk by 2.0-3.7 times and lowers the onset age by 10 years. A risk assessment software tool, PancPRO, has been generated and is available for calculating the risk for individuals with familial pancreatic cancer (http://www4.utsouthwestern.edu/breasthealth/cagene/default.asp)[35].

Screening modalities and the current screening programs: Many of the screening programs have used additional investigational biomarker to complement imaging tests to identify the early lesions. A commonly used marker, CA19-9, is neither sensitive nor specific independently for early PDAC or precursor detection. Kim *et al*[63] in their study found only 0.9% positive-predictive value using the standard cut-off value (37 U/mL). Other biomarkers investigated recently include MIC-1, CEACAM-1, SPan1, DUPAN, Alpha4GNT, and PAM4, but none is validated for routine clinical use[64]. In another approach, elevated fasting-glucose level has been demonstrated to be associated with sporadic PDAC[65] and is currently utilized by an European registry in high-risk people with mutational analysis of pancreatic juice along with *p16* promoter methylation status.

Several international screening programs exist for PDAC in high-risk individuals. "Cancer of the Pancreas Screening Study" (CAPS study), led by John Hopkins University, is the largest screening program that involves 24 American Centers of Excellence. To date, three studies, CAPS 1, CAPS 2 and CAPS 3, have been completed (Table 2).

Table 2 Results of screening programs for pancreatic cancer in high-risk groups *n* (%)

Study	CAPS1	CAPS2	CAPS3	U of Washington	FaPaCa	Dutch Study
Diagnostic Yield	2 (5.3)	8 (10)	92 (43)	10 (13)	1 (1.3)	10 (23)

Positive finding of abnormal imaging such as mass (solid, cyst) or abnormal duct in the cases. CAPS: Cancer of the Pancreas Screening Study; FaPaCa: Familial Pancreatic Cancer Study.

In the CAPS 1, thirty-eight patients including 31 from a kindred with > 3 affected with PDAC, 6 with 2 affected relatives, and 1 patient with Peutz-Jeghers syndrome (PJS) were studied. Screening protocol with EUS revealed six pancreatic masses: 1 invasive PDAC, 1 benign IPMN, 2 serous cystadenomas, and 2 non-neoplastic lesions. The yield of screening was 5.3% in this study[66]. In the CAPS 2, seventy-eight high-risk patients were studied[67]. In 8 patients, the screening found pancreatic neoplasia, confirmed by surgery or FNA: 6 benign IPMNs, 1 IPMN that progressed to invasive PDAC, and another had high-grade pancreatic intraepithelial neoplasia (PanIN-3). The CAPS 3 was a multicenter prospective cohort study involving annual EUS, MRI screening with assays of DNA and protein markers in serum and pancreatic juice. Over 200 patients were enrolled over a three-year period. The study results on the detection modality comparison demonstrated that the EUS has the highest rate of detection of early neoplastic changes in up to 42.6% of the asymptomatic high-risk group[68].

In another study from the University of Washington, high-risk familial cohorts were screened with EUS, beginning 10 years prior to the earliest index PDAC case. If EUS was normal, they underwent a repeat EUS in 2-3 years. With abnormal EUS findings, they were referred for ERCP and if abnormalities were noted, patients were offered surgical intervention[69]. Among 75 screened subjects, 15 had gone to surgery for abnormal EUS and ERCP findings. All surgical cases revealed premalignant lesions: PanIN-3 in 10 and PanIN-2 in five cases[70]. The study gave a yield of 13% (10 out of 75) for detecting PanIN-3 lesions. A single patient developed unresectable PDAC during the surveillance.

In a German PDAC screening program (FaPaCa), 76 patients were followed using annual EUS, MRCP, and laboratory assays (*CDKN2a* and *BRCA2* genetic analysis, CA19-9 and CEA). Any appreciable lesion was evaluated with EUS. With an abnormal finding, the patient underwent surgical exploration and if malignancy was detected, total pancreatectomy was performed. In 10 cases, lesions were seen on EUS as compared to only seven detected by MR scan. Out of the seven MRCP-positive cases, six underwent resections and the histology showed one PanIN-3, one PanIN-2, one PanIN-1, and three with other benign lesions. This resulted in a diagnostic yield of 1.3% for PanIN-3 detection[71]. Another study from the Netherlands in 44 high-risk subjects demonstrated a 7% detection rate for asymptomatic PDAC and a 16% for premalignant lesions[72].

The International CAPS Consortium have recently met and reported a suggested guideline for current PDAC screening based on the risk[73]. A consensus (⩾ 75% agreement by the participants) was reached that the following groups should be offered screening (only to individuals who are surgical candidate): (1) FDRs of the cancer patients from a familial pancreatic cancer cohort with at least two affected FDRs; (2) patients with Peutz-Jeghers syndrome; and (3) *p16*, *BRCA2* and hereditary non-polyposis colorectal cancer mutation carriers with at least single affected FDR. The initial screening should include EUS and/or MRI. However, consensus was not reached on the beginning and the end age of screening/surveillance and the interval of the examination. Their conclusions also included requirement for further studies, and the clinical management should occur at high-volume centers with multidisciplinary teams.

FUTURE OF PANCREATIC CANCER SCREENING

Current screening programs have demonstrated that the EUS evaluation can detect premalignant lesions and early cancers in certain small subset of high-risk groups. However, as the overwhelming majority of PDAC cases involve patients who develop the disease sporadically without a recognized genetic abnormality, the application of this modality for PDAC detection screening is very limited for the general adult population.

Select population based approach

Identification of a higher-PDAC-risk group: As the prevalence of PDAC in the general United States population over the age 55 is approximately 68 per 100000, a candidate discriminant test with a specificity of 98% and a sensitivity of 100% would generate 1999 false-positive test results and 68 true-positives[74]. Thus, relying on a single determinant for distinguishing the PDAC early-stage cases from the general population would necessitate a highly accurate test with a specificity of greater than 99%. More practical approach, then, would be to begin with a subset of population with a higher prevalence, and in conjunction with novel surrogate markers to curtail the at-risk subset, we could begin to identify the

group with significantly increased PDAC risk for whom the endoscopic/imaging-based screening strategy could be applied.

An initial approach in selection of the screening population is to utilize selective clinical parameters that could be used to curtail the subset of the general population at increased PDAC risk. For instance, based on the epidemiological evidence, such clinical parameters include hyperglycemia or diabetes, which are noted in 50%-80% of pancreatic cancer patients[75-79]. Though not encompassing all PDAC patients, this subset includes a much larger proportion of PDAC patients for whom we may select further for screening. Similarly, patients with a history of chronic pancreatitis or obesity are reported to have increased PDAC risk during their lifetime[80-85]. Recent findings from molecular biology and animal studies investigating effects of diet-induced obesity in a PDAC mouse model demonstrated increased occurrence of pancreatic inflammation and accelerated pancreatic neoplastic changes, supporting the association of obesity and pancreatic inflammation and PDAC risks[86,87]. Considering that millions are being diagnosed with diabetes or glucose intolerance, chronic pancreatitis, or obesity annually in comparison to PDAC, however, further refinement of the screening patient group is critically needed to justify for developing a larger scale screening protocol.

Translational research - application of metabolomics approach

Initially established as a key methodology in the field of inborn metabolic errors and toxicology, metabolomics have developed over the years to examine a much wider array of low-molecular-weight products or intermediates within the biological state of a cell, tissue, organ, or organism. A metabolome represents a physiological readout of the biochemical state in an individual's body compartment, and provides the functional terminal signals of the genome and proteome, reflecting more closely the current phenotypic state of an individual in response to the environmental stimuli[88]. Thus, metabolomics data has considerable potential in elucidating cancer-development risks, with its additional capacity for providing temporal molecular information to the ongoing changes originating from genetic PDAC risk alone.

With the recent advancement in the technology and resumed interest in the cancer-associated metabolic abnormality[89,90], application of metabolomics in the cancer field has attracted more attention[91]. Cancer-related metabolic reprogramming, Warburg effect, has been known since nearly a century ago in association with various solid tumors including PDAC[92], as cancer cells undergo energetically inefficient glycolysis even in the presence of oxygen in the environment (aerobic glycolysis)[93]. A number of common cancer mutations including Akt1, HIF (hypoxia-inducible factor), and p53 have been shown to support the Warburg effect through glycolysis and down-regulation of metabolite flux through the Krebs cycle[94-101]. In PDAC, increased phosphorylation or activation of Akt1 has also been reported (illuminating on the importance of enzyme functionality)[102] as well as involvement of HIF1 in the tumor growth *via* effects on glycolytic process[103,104] and membrane-bound glycoprotein (MUC17) regulation[105] - reflective of activation of metabolic pathways. Further evidences of loss-of-function genetic mutations in key mitochondrial metabolic enzymes such as succinate dehydrogenase and fumarate hydratase, isocitrate dehydrogenase, phosphoglycerate dehydrogenase support carcinogenesis and the Warburg effect[106-110]. Other important alternative pathways in cancer metabolism such as glutaminolysis and pyruvate kinase isoform suppression have been shown to accumulate respective upstream intermediates and reduction of associated end products such as NADPH, ribose-5-phosphate and nucleic acids[111-116]. As such, various groups have reported metabolomics biomarker applications for different cancers[117,118].

As a major organ involved in metabolic regulation in a healthy individual, pancreatic disorder such as malignancy is anticipated to influence the normal metabolism, presenting further rationale and interest in elucidating the implication of malignant transformation and PDAC development. Proteomic analysis of the pancreatic cancer cells demonstrated alteration in proteins involved in metabolic pathways including increased expression of glycolytic and reduced Krebs cycle enzymes, and accumulation of key proteins involved in glutamine metabolism, in support of Warburg effect. These in turn play significant role in nucleotide and amino acid biosynthesis required for sustaining the proliferating cancer cells[119]. Applications of sensitive mass spectrometric techniques in metabolomics study of PDAC detection biomarkers have led to identification of a set of small molecules or metabolites (or biochemical intermediates) that are potent discriminants of developing PDAC and the controls (See Figure 1 as an example of metabolomics based analysis, allowing segregation of PDAC from benign cases). Recent reports from our group as well as others have demonstrated that specific candidate metabolites consisting of amino acids, bile acids, and a number of lipids and fatty acids - suspected to be reflective of tumor proliferation as well as many systemic response yet to be determined - were identified as potential discriminant for blood-based PDAC biomarkers[120-123]. As a further supporting data, elucidation of lipids and fatty acids as discriminant factors from PDAC and benign lesions from the cancer tissue and adjacent normal tissue has been reported recently[124].

By virtue of simultaneously depicting the multiple

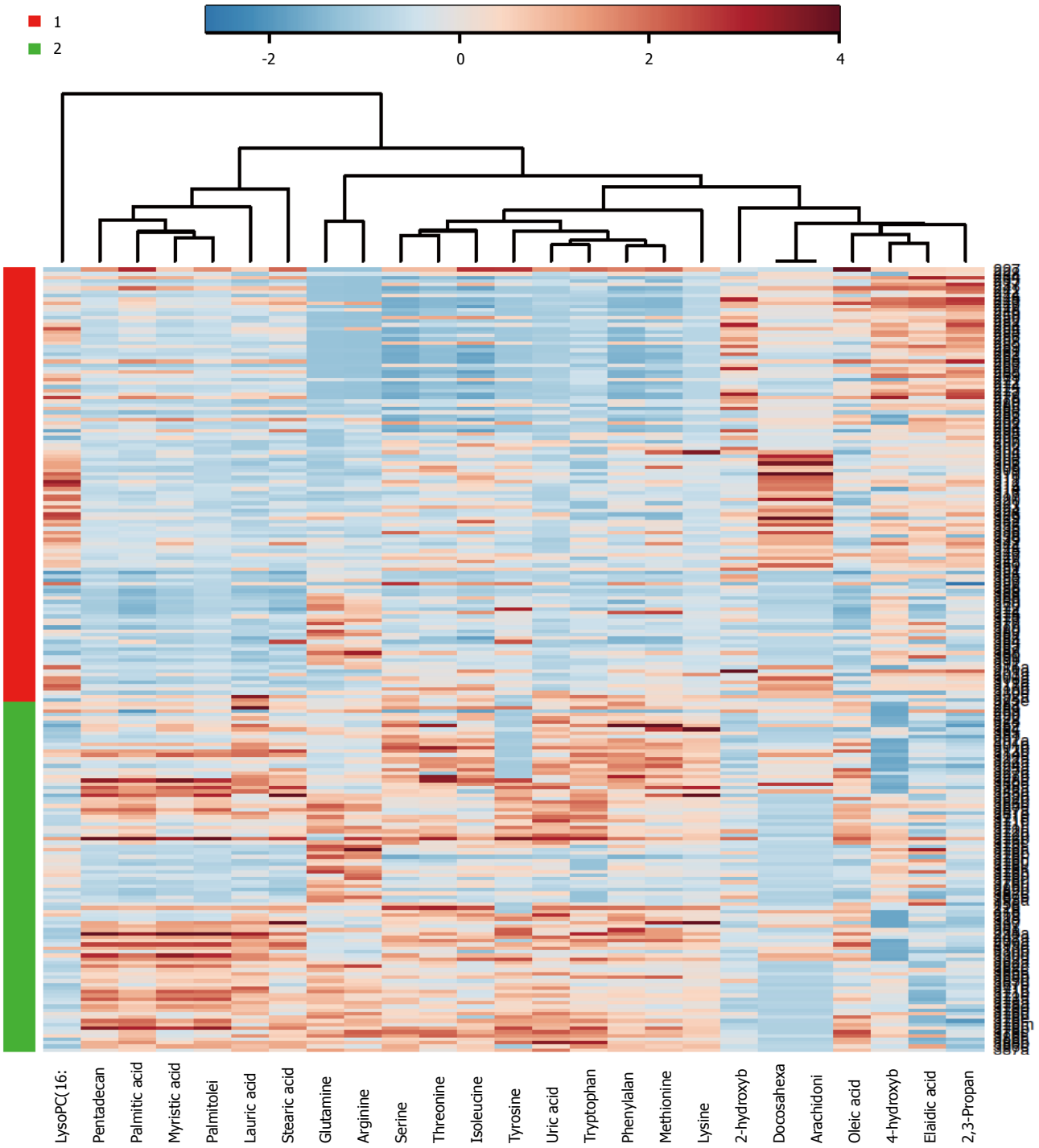

Figure 1 Example of metabolomics based analysis, allowing segregation of pancreatic ductal adenocarcinoma from benign cases. Heat map illustration of discriminant capability of a metabolite set derived from gas chromatography and liquid chromatography/mass spectrometry plasma metabolomics dataset comparing pancreatic ductal adenocarcinoma patients (Red or group 1; *n* = 110) and benign pancreas (includes benign cysts, chronic pancreatitis, and normal pancreas) (Green or group 2; *n* = 90). Metabolites are plotted on x-axis, and the cases on the y-axis. Blue color indicates data points with a value smaller than the median of the respective metabolite and the red indicates higher values. This candidate set of metabolites enabled the segregation of the two groups.

metabolite levels, metabolomics approach reveals various biochemical pathways that are uniquely involved in malignant conditions and has led to findings such as abnormalities of glycine and its mitochondrial biosynthetic pathway, as a potential therapeutic target in certain cancers[125]. Moreover, in combination with other systems biology approaches such as transcriptomics and proteomics, further refinement in characterization of cancer development and therapeutic targets as well as identification of potential biomarkers could be realized for PDAC. Since many enzymes in a metabolic network determine metabolites' level and nonlinear quantitative relationship from the genes to the proteome and me-

tabolome levels exist, a metabolome cannot be easily decomposed to a specific single marker, which will designate the cancer state[126]. Thus, in order to delineate a pathological state such as PDAC, multiple metabolomic features might be required for accurate depiction of a developing cancer. Future studies are anticipated to incorporate cancer systems' biological knowledge, including metabolomics, for optimal designation of PDAC biomarkers, which would be utilized in conjunction with a clinical-parameter-derived population subset for establishing the PDAC screening population. Subsequently, further validation studies for the PDAC biomarkers need to be performed.

CONCLUSION

Current imaging-based detection and diagnostic methods for PDAC is effectively providing answers to clinical questions raised for patients with signs or symptoms of suspected pancreatic lesions. However, the endoscopic/imaging-based screening schemes are currently limited in applications to early PDAC detection in asymptomatic patients, aside from a small group of known genetically high-risk groups. There is a high demand for developing a method of selecting distinct subsets among the general population for implementing the endoscopic/imaging screening test effectively. Application of combinations of clinical risk parameters/factors with the developing molecular biomarkers from translational science such as metabolomics analysis brings hopes of providing us with early PDAC detection markers, and developing effective early detection screening scheme for the patients in the near future.

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REVIEW

Vitamin D: A new player in non-alcoholic fatty liver disease?

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Abstract

Vitamin D through its active form 1a-25-dihydroxyvtamin D [1,25(OH)$_2$D] is a secosteroid hormone that plays a key role in mineral metabolism. Recent years have witnessed a significant scientific interest on vitamin D and expanded its actions to include immune modulation, cell differentiation and proliferation and inflammation regulation. As our understanding of the many functions of vitamin D has grown, the presence of vitamin D deficiency has become one of the most prevalent micronutrient deficiencies worldwide. Concomitantly, non-alcoholic fatty liver disease (NAFLD) has become the most common form of chronic liver disease in western countries. NAFLD and vitamin D deficiency often coexist and epidemiologic evidence has shown that both of these conditions share several cardiometabolic risk factors. In this article we provide an overview of the epidemiology and pathophysiology linking NAFLD and vitamin D deficiency, as well as the available evidence on the clinical utility of vitamin D supplementation in NAFLD.

Key words: Steatohepatitis; Non-alcoholic fatty liver disease; Fatty liver; Vitamin D

Core tip: Non-alcoholic fatty liver disease (NAFLD) is a multifactorial disease and its pathogenesis is closely linked to the metabolic syndrome. Vitamin D deficiency, which also shares similar associations with obesity and sedentary lifestyle, is often found together with NAFLD. As our understanding of the many functions of vitamin D has grown, emerging evidence points to a closely linked and potentially causative relationship between vitamin D deficiency and NAFLD. As such, vitamin D is now emerging as an immunomodulatory and anti-fibrotic agent. However, in order to implement clinical recommendations larger, randomized, placebo-control trials are required to better evaluate the efficacy of vitamin D replacement in NAFLD.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) has become the most common form of chronic liver di-

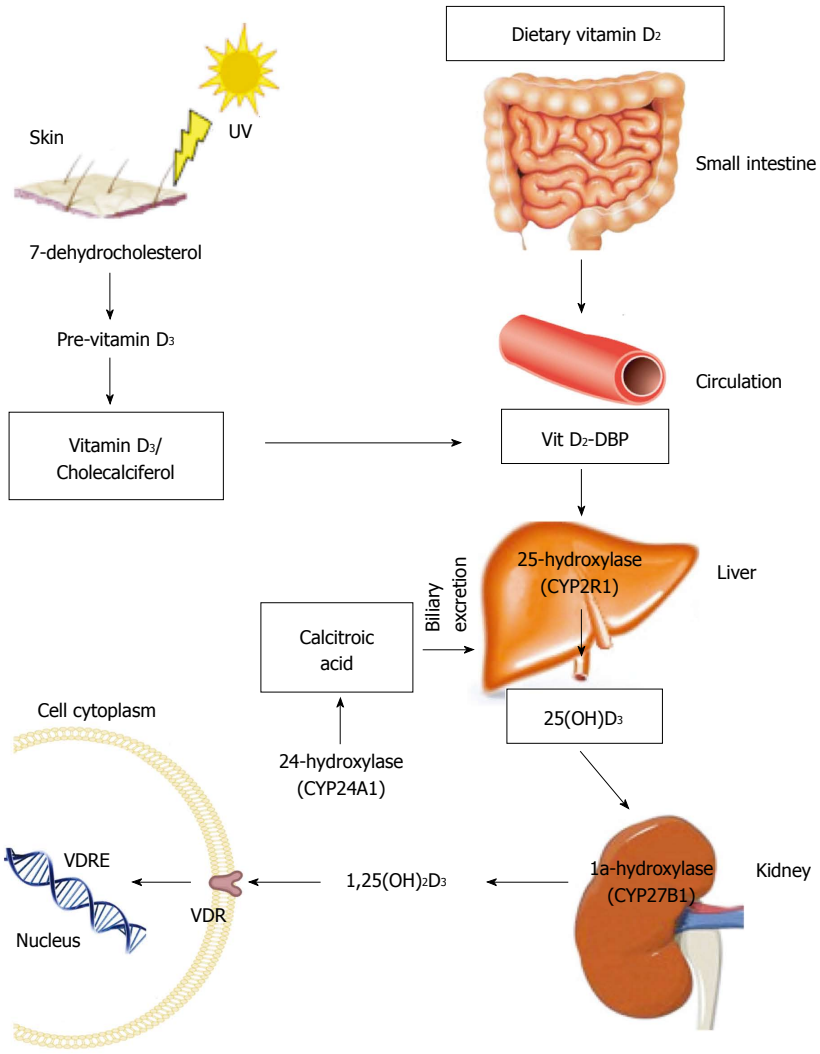

Figure 1 Vitamin D synthesis and metabolism. DBP: D binding protein; VDRE: Vitamin D response element.

sease in Western countries with prevalence as high as 30%[1,2], thus exceeding that of viral hepatitis and alcoholic liver disease. NAFLD represents a continuum of hepatic injuries, which progress from simple fatty liver to steatohepatitis (NASH), cirrhosis or even hepatocellular carcinoma. The metabolic syndrome is universally considered as the key factor in the pathogenesis of NAFLD[3,4]. However, the evolution of liver inflammation in NAFLD and the progression from simple fatty liver to steatohepatitis and hepatic fibrosis is more complex[5]. As our understanding in the pathogenesis of NASH continues to evolve, vitamin D is emerging as an important player in the development and progression of NAFLD. This review will assess the role of vitamin D deficiency in the pathogenesis of NAFLD, explore the epidemiologic evidence that supports a link between vitamin D deficiency and NAFLD and provide available evidence on the clinical utility of vitamin D replacement in NAFLD subjects.

VITAMIN D METABOLISM

Vitamin D is a fat-soluble vitamin. Although, multiple forms of this vitamin exist, vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol) are the two major forms. Vitamin D_2 is produced by some organisms of phytoplankton, invertebrates and yeast in response to ultraviolet irradiation but it is not constitutively produced by vertebrates. Thus this form of vitamin D has been exploited commercially and is used for fortification and supplementation. Vitamin D_3 on the other hand, originates in the skin of most vertebrates including humans, after irradiation of 7-dehydrocholesterol with ultraviolet light (UVB) (Figure 1). Dietary vitamin D_2 is absorbed by the small intestine and incorporated into chylomicrons where it is transported to the liver bound to vitamin D-binding protein. In the liver, vitamin D from both the skin and diet is then metabolized by 25-hydroxylase (CYP2R1) to 25-hydroxyvitamin D [25(OH)D], which

is the major circulating metabolite and the most widely used indicator of vitamin D stores. 25(OH)D is transported to the kidney where it undergoes hydroxylation by 1a-hydroxylase (CYP27B1) to the biologically active form 1a,25-dihydroxyvitamin D [1,25(OH)$_2$D]. Finally, *via* binding to vitamin D receptor (VDR), 1,25(OH)$_2$D is able to exert its biological actions.

The synthesis of 1,25(OH)$_2$D is tightly regulated by the synthetic activity of 1a-hydroxylase and the catabolic activity of 24-hydroxylase (CYP24A1) which catabolizes 1,25(OH)$_2$D to the water soluble and biologically inactive calcitoic acid which is then excreted in the bile. Parathyroid hormone, 1,25(OH)$_2$D and Fibroblast growth factor 23 (FGF23) are the main regulators of these enzymes. 1,25(OH)$_2$D decreases its own synthesis though negative feedback but also by way of inhibition of parathyroid hormone (PTH) which is the main stimulus for 1a-hydroxylase transcription. Fibroblast growth factor 23, secreted from osteoblasts, acts on the kidneys to suppress renal expression of 1a-hydroxylase and to promote 24-hydroxylase activity which result in reduced production of 1,25(OH)$_2$D.

VITAMIN D TARGETS

The leading and most widely known physiological function of 1,25(OH)$_2$D is to regulate mineral and skeletal homeostasis. However, over the last decades the functions of vitamin D have been broadened beyond those on skeletal tissue and calcium homeostasis. Indeed, the finding of VDR expression in a wide range of tissues such as the immune system (T and B cells, macrophages, and monocytes), the reproductive system (uterus, testis, ovary, prostate, placenta, and mammary glands), the endocrine system (pancreas, pituitary, thyroid and adrenal cortex), in muscles (skeletal, smooth and heart muscles), and in brain, skin, and liver has stimulated considerable interest in understating the putative pleiotropic properties of vitamin D and introduced the idea of a paracrine/autocrine role in regulating cell proliferation, differentiation and apoptosis as well as immune-cells regulation[6,7].

VITAMIN D DEFICIENCY AND NAFLD: THE EPIDEMIOLOGIC EVIDENCE TO DATE

Numerous publications propose that low levels of 25(OH)D are strongly associated with features of the metabolic syndrome[8,9] and may play an important role in modifying the risk for cardio-metabolic outcomes including Type 2 diabetes (T2DM), hypertension and cardiovascular disease[10]. A recent systematic review found that 25(OH)D levels > 25 ng/mL were associated with 43% lower risk of T2DM compared to levels < 14 ng/mL[11]. In the same study, vitamin D treatment improved insulin resistance among patients with baseline glucose intolerance. Similarly, another meta-analysis showed that vitamin D supplementation improves insulin resistance compared to placebo, albeit the effect was weak[12]. In support of the beneficial role of vitamin D in diabetes and insulin resistance are the findings of various animal studies showing that lack of VDR in mice or vitamin D deficiency impairs insulin secretion from pancreatic beta cells[13]. In contrast to the above findings, are the results of a recent meta-analysis by Seida *et al*[14]. In this study, 35 randomized controlled trials were examined with a total of 43407 patients. No significant effect of vitamin D supplementation on the prevention of diabetes in individuals without diabetes, or on the reduction of insulin resistance and hyperglycemia in those with pre-diabetes or established type 2 diabetes was found. However these results are limited by the presence of moderate heterogeneity between the studies, the associated risks of bias and the short term of follow up.

Given the strong association of NAFLD with obesity and the metabolic syndrome, recent years have witnessed a significant scientific interest into the potential role of vitamin D in NAFLD. Accumulating epidemiological data suggest that low levels of serum 25(OH)D are associated with NAFLD as diagnosed either by biochemistry, imaging or biopsy. These data are summarized in a recently published meta-analysis in which NAFLD subjects were 26% more likely to be vitamin D deficient compared to controls[15]. In US the largest of these studies was by Liangpunsakul *et al*[16] in which the authors reported that in a subset of 1287 adult participants from the NHANES Ⅲ database, those with unexplained elevation in serum alanine aminotransferase (ALT) levels - a proxy of NAFLD - had lower 25(OH)D levels than those with normal ALT levels (24.7 ± 10.4 ng/mL *vs* 26.8 ± 10.9 ng/mL, $P < 0.01$). Compared to the lowest quartile, patients with the two top quartiles of serum 25(OH)D levels had significantly lower prevalence of unexplained elevation in serum ALT, independently of metabolic syndrome features[16]. In Asia, the largest study was a cross-sectional study of 6567 Korean men which found that subjects in the lowest tertile of 25(OH)D levels had a significantly increased risk for NAFLD compared to thosein the highest tertile, even after adjusting for body mass index and metabolic syndrome (OR = 1.247 and 1.408 *vs* the highest tertile, $P < 0.001$)[17]. A more recent Korean study however, showed contradictory results. In this study the authors analyzed data from the Korean National Health and Nutrition Examination database (KNHANES IV) with more than 16000 individuals and found that obese subjects (BMI > 25) with > 2 components of the metabolic syndrome were more likely to have elevated liver enzymes compared to normal weight subjects; however there was no

Table 1 Potential mechanisms and evidence to support a benefit for vitamin D in non-alcoholic fatty liver disease

Mechanisms	Evidence
Improvement in insulin secretion and insulin resistance	Presence of VDR in pancreatic beta cells[26] Expression of 1-α-hydroxylase enzyme in pancreatic beta cells[79] Impaired insulin secretory response in mice lacking a functional VDR[13] Transcriptional activation of the human insulin gene by 1,25(OH)$_2$D[80] Vitamin D deficiency impairs glucose-mediated insulin secretion from rat pancreatic beta cells *in vitro*[81] and *in vivo*[82] Vitamin D enhances insulin responsiveness for glucose transport in muscle cells[83] Vitamin D up-regulates glucose transporter 4 (GLUT4) translocation and glucose utilization in adipocytes[44]
Improvement in adipose tissue inflammation	Higher 25(OH)D concentrations were independently associated with higher adiponectin concentrations in a large cohort of men and women[40] Reduction of IL-6 in adipocytes after supplementation of vitamin D in mice fed high fat diet[84] 1,25-dihydroxyvitamin D treatment in human adipocytes inhibits NF-κB pathway and reduces pro-inflammatory cytokine release[85,86] 1,25-dihydroxyvitamin D inhibits macrophage recruitment and increases adiponectin expression in preadipocytes[87]
Improvement in hepatic inflammation	Vitamin D deficiency causes TLR activation and exacerbates hepatic inflammation[42] Artificial sunlight therapy in rats reduced liver inflammation and apoptosis[43] VDR expression on cholangiocytes was inversely correlated with steatosis severity and nonalcoholic fatty liver disease score in NASH patients[31]
Improvement in hepatic fibrosis	Presence of VDR in HSC[24] Vitamin D treatment suppresses HSC proliferation in cultured HSC from rats[73] Vitamin D treatment downregulates pro-fibrotic marker TIMP-1 and collagen type Ⅰ production in cultured HSCs[73,74] VDR knockout mice develop spontaneous liver injury with fibrosis[30]

VDR: Vitamin D receptor; TLR: Toll-like receptor; IL: Interleukin; TLR: Toll like receptor; NASH: Nonalcoholic steatohepatitis; HSC: Hepatic stellate cells.

significant difference in vitamin D levels between the groups[18].

Targher *et al*[19] was the first to study the association between biopsy-proven NAFLD and vitamin D levels. The study confirmed that 25(OH)D concentrations were lower in NAFLD subjects compared to matched controls. Furthermore 25(OH)D levels predicted the histological severity of NAFLD, with NASH patients having lower 25(OH)D levels compared to those with isolated fatty liver. These findings have been confirmed by four other studies with biopsy-proven NAFLD in adults[20,21] and in children[22,23].

Collectively, the data from the published studies indicate that NAFLD subjects are more likely to be vitamin D deficient compared to controls. However, definite directionality of the results cannot be ascertained due to the nature of the above studies (*i.e.*, cross-sectional) and the limitations observed which include the variability in the method of diagnosis of NAFLD, clinical heterogeneity among the study groups and variability in defining vitamin D deficiency.

NAFLD PATHOPHYSIOLOGY

Our understanding of the pathogenesis of NAFLD has evolved from the relatively simplistic "two-hit" hypothesis to the "multiple-hits" hypothesis[5]. In this model, a number of diverse, parallel processes contribute to the development and progression of liver inflammation from simple hepatic steatosis to steatohepatitis and hepatic fibrosis. A number of these pathways can be affected by vitamin D and relate to the hormonal, immunologic and cellular differentiation "non-classical" effects of vitamin D. Below, we will discuss each of these pathways. A summary of evidence is provided in Table 1 and Figure 2 provides a schematic representation of these mechanisms.

PUTATIVE MECHANISMS LINKING VITAMIN D DEFICIENCY TO NAFLD

Vitamin D signaling

Vitamin D mediates its intracellular signals via its receptor VDR which is constitutively expressed in the liver[24,25]. It has been estimated that VDR regulates over 200 genes involved in glucose and lipid metabolism[13,26], inflammation[27], cellular proliferation and differentiation and apoptosis[28]. In a GWAS study of NAFLD subjects, four single nucleotide polymorphisms (SNPs) were found to have significant association with NAFLD. Among these four SNPs *GC* gene was included which is predominately expressed in hepatocytes and codes for vitamin D binding protein, the main carrier protein for vitamin D[29]. We also know from animal studies that normal functioning of VDR is crucial for liver fibrosis, as VDR knockout mice exhibit spontaneous liver injury with fibrosis[30]. In humans, Barchetta *et al*[31] showed that liver VDR expression is inversely correlated with severity of NAFLD on histopathology, independently

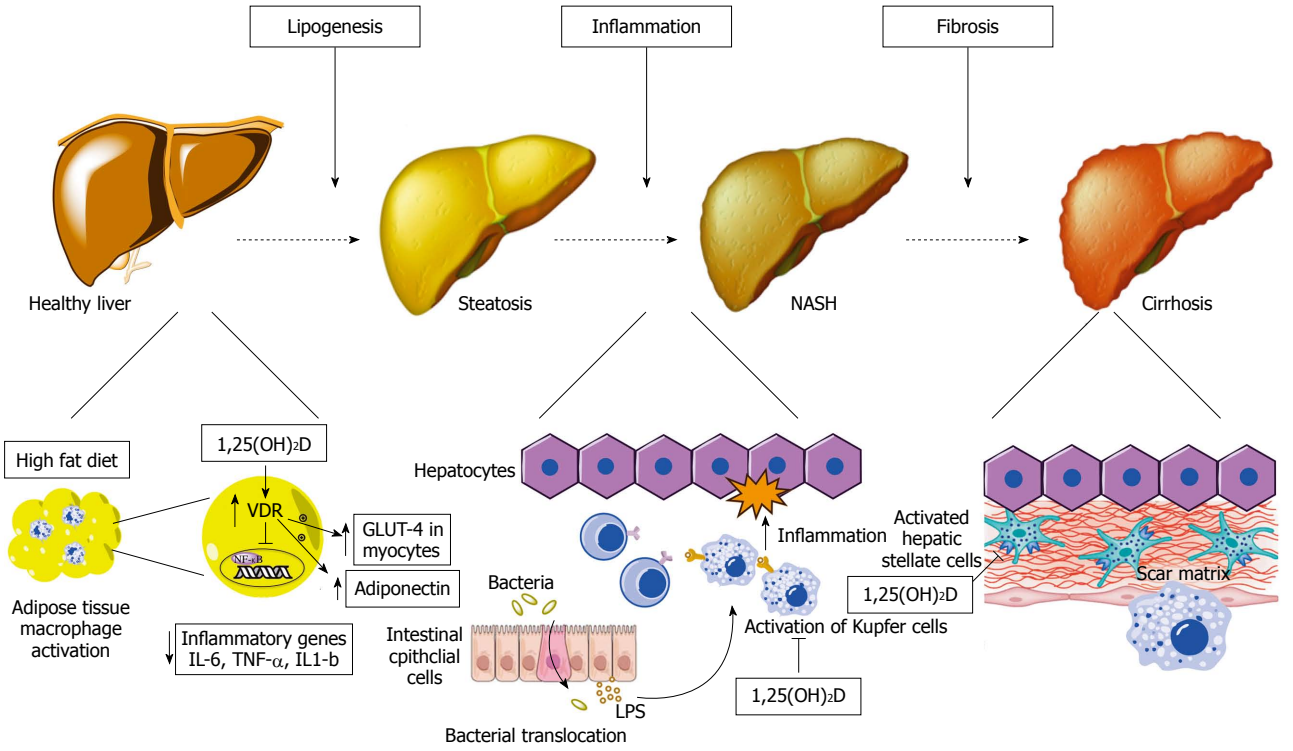

Figure 2 Schematic representation of metabolic, anti-inflammatory and anti-fibrotic effects of vitamin D on hepatocytes and non-parenchymal hepatic cells (hepatic stellate cells, Kupffer cells) in non-alcoholic fatty liver disease. Left: At the initial stage of lipogenesis, 1,25(OH)$_2$D acts on adipocytes and inhibits NF-κB transcription, known as the pro-inflammatory "master switch", and thus inhibits the expression of the inflammatory cytokines IL-6, TNF-α and IL-1β. It also increases adiponectin secretion from adipoycytes and enhances GLUT-4 receptor expression in myocytes, both of which improve insulin resistance; Middle: Increased gut permeability allows translocation of bacterial pathogens which can activate Toll like receptors on Kupffer cells. 1,25(OH)$_2$D downregulates the expression of TLR-2, TLR-4 and TLR-9 in these cells and thus ameliorates inflammation; Right: 1,25(OH)$_2$D acts on hepatic stellate cells by binding to VDR and reduces proliferation of these cells that play a major role in inducing fibrosis. VDR: Vitamin D receptor; TLR: Toll like receptor; LPS: Lipopolysacharides.

from other metabolic parameters such as BMI, insulin resistance or adiponectin.

Adipose tissue, insulin resistance and hepatic inflammation - the role of vitamin D

Insulin resistance, a key risk factor in the pathogenesis of NAFLD, is linked to the development of oxidative stress and lipotoxicity[32,33]. As a result, hepatic steatosis resulting from accumulation of free fatty acids is associated with a state of chronic hepatic inflammation. An important mediator in this process is nuclear factor κ-β (NF-κB) that functions as a pro-inflammatory "master switch" by upregulating the transcription of a wide range of inflammatory mediators. Accordingly, increased NF-κB activity in the livers of high fat diet mice is associated with the increased expression of pro-inflammatory cytokines, including TNF-α, IL-6, IL-1β and activation of Kupffer cells[34]. These cytokines are capable of producing all of the classical histological features of NASH including hepatocyte necrosis/apoptosis, neutrophil chemotaxis and activation of hepatic stellate cells. Moreover, human studies have demonstrated increased cytokine gene expression in the livers of patients with NASH compared to obese controls with normal livers, with the increased expression correlating with histolocical severity[35]. Adiponectin on the other hand, has been described as the prototypic adipokine by way of its function as an anti-inflammatory agent. In murine models, high levels of adiponectin have been experimentally shown to decrease necroinflammation and steatosis in alcoholic and nonalcoholic fatty liver disease[36] as well as improved insulin resistance[37]. Moreover, studies in humans showing reduced serum levels of adiponectin and reduced hepatic expression of its receptor in patients with NASH compared with body mass index-matched patients with steatosis[38,39], provide strong supportive evidence that reduced adipocyte production of adiponectin plays an important role in the pathogenesis of progressive NAFLD.

The role of vitamin D in adipokine activity is an active area of research. Vaidya *et al*[40] showed a positive association between 25(OH)D concentrations and levels of adiponectin in a large cohort of patients. Interestingly, this relationship was independent of BMI. Furthermore, in a double-blind, randomized, controlled trial of Iranian type 2 diabetic patients, daily intake of 1000 IU vitamin D either with or without extra calcium for 12 wk resulted in a significant increase of serum adipokines including adiponectin and decreased cellular secretion of the inflammatory cytokines IL-6 and IL-1β[41].

Additional evidence from animal studies further support the notion of an immunomodulatory role of

vitamin D in NAFLD. To explore the effect of vitamin D deficiency on inflammatory markers Roth *et al*[42] used a rat model consistent of 4 groups; rats were fed either a low-fat diet alone (LFD) or with vitamin D depletion (LFD + VDD) or high-fat Western diet which was either replete (WD) or deficient in vitamin D (WD + VDD). In VDD groups, blood 25(OH)D levels were reduced compared to the replete diet groups. Mice fed WD/VDD showed increased hepatic steatosis comprared to LFD groups. Liver histology also showed increased lobular inflammation and NAFLD activity score in WD/VDD group compared to the WD alone group. In addition WD/VDD mice had increased hepatic mRNA levels of resistin, IL-4, IL-6 and TNF-α markers known to be implicated in oxidative stress and hepatic inflammation. Accordingly in another rat NASH model[43], phototherapy increased 25(OH)D and 1,25(OH)$_2$D levels while reducing hepatocyte inflammation, fibrosis and apoptosis compared to controls. Phototherapy also improved insulin resistance and increased serum adiponectin in association with reduced hepatic expression of inflammatory genes TNF-α and TFG-β. In total, these findings suggest that vitamin D deficiency worsens NAFLD related to upregulation of hepatic inflammatory and oxidative stress genes.

Another interesting and plausible mechanism underlying the association of diabetes/insulin resistance with low vitamin D levels has been recently showed in an *in-vitro* study where 3T3L1 adipocytes were treated with high glucose in the presence or absence of 1,25-dihydroxyvitamin D. 1,25(OH)$_2$D treatment of adipocytes caused significant up-regulation of GLUT4 receptor expression and its translocation to cell surface, and an increase in glucose uptake as well as glucose utilization[44]. Supplementation also stimulated adiponectin secretion in high glucose-treated cells, lending further weight of a beneficial effect of vitamin D in reducing adipose tissue inflammation.

Intestinal microbiome and innate immunity in NAFLD and the role of viatamin D deficiency

The liver is positioned between the gut and systemic circulation and in addition to its synthetic function it has a key role in degrading and removing toxins, exogenous antigens, and infectious agents responding to exogenous antigenic molecules. This role makes the liver not only a metabolic organ but also a mediator of systemic and local innate and adaptive immunity[45]. The intestinal epithelial cells prevent the translocation of bacterial products to the portal circulation. When this barrier is ineffective the liver cells are exposed to bacterial products and this translocation may impair liver homeostasis and trigger liver inflammation, inducing the innate immune response[46,47]. A study from Italy showed that patients with NAFLD have increased gut permeability and small intestinal bacterial overgrowth (SIBO). Both gut permeability and the prevalence of SIBO correlated with the severity of steatosis but not with presence of NASH in this study[48].

Bacterial lipopolysaccharides (LPS) are activators of the immune system and in animal models were involved in the development of both systemic inflammation and obesity[49]. Toll Like receptor-4 (TLR-4) recognizes a diverse array of pathogen associated molecular patterns including LPS[50]. The role of TLR-4 has been studied and there is a clear association between TLR-4 activation and NAFLD[48,49,51-54]. Interestingly, the development and progression of NAFLD by western diet is exacerbated by vitamin D deficiency through the activation of TLR2 and TLR4 by way of CD14/LBP, and stimulation of downstream inflammatory signaling molecules leading to steatosis and inflammation[42].

Toll-like receptor 5 (expressed in the gut mucosa helping defense against infection) is implicated in the development of metabolic syndrome and alterations in gut microbiota. In a study published in 2010, TLR-5-deficient mice developed hyperphagia, obesity, insulin resistance and hepatic steatosis. In this study, transfer of microbiota from TLR-5-deficient mice to healthy mice led to development of *de novo* disease, indicating a possible connection between TLR-5 and intestinal microbiota in NAFLD[55]. In contrast to this study, Kanuri *et al*[54] found that TLR-5 is significantly overexpressed in patients with NAFLD compared to controls thus making the role of TLR-5 in the development of NAFLD unclear.

A different subtype of TLR, TLR-9 is activated by bacterial/viral DNA and has been implicated in the development of steatohepatitis in animal models[56]. Miura *et al*[57] showed that TLR-9-deficient mice failed to develop inflammation *vs* controls when exposed to IL-1b. In another study mentioned earlier the investigators showed that vitamin D deficient mice following Western Diet had increased levels of messenger RNA of TLR-2, TLR-4, and TLR-9[42]. However, there are no randomized controlled trials studying whether vitamin D replacement is beneficial in suppressing the effects of TLR-4 and TLR-9.

The role of the major components of the innate immunity like macrophages and Kuppfer cells[58-61], neutrophils[62-64], eosinophils[65] and dendritic cells (DC)[66,67] in the pathogenesis of NAFLD and insulin resistance has been studied but a detailed report extends beyond the goals of the current review. It is however interesting to note the immunomodulatory effect of vitamin D in DC. Dendritic cells express VDR[68] and treatment with 1,25(OH)$_2$D inhibits DC maturation and promotes a tolerogenic phenotype in some studies[69,70]. However Henning *et al*[66] showed that DC depletion markedly exacerbates intrahepatic fibroinflammation. Thus the role of vitamin D in the

regulation of this pathway is not clear yet.

Vitamin D and hepatic fibrosis

The development of liver fibrosis in NAFLD indicates advanced disease and is in fact the strongest predictor for disease-specific mortality[71]. It is characterized by an accumulation of extracellular matrix (ECM) with subsequent destruction of the normal liver architecture, leading to liver cell dysfunction. Hepatic stellate cells (HSCs) play a critical role in the development of liver fibrosis, since they are responsible for excessive deposition of ECM proteins, predominantly type Ⅰ collagen. Two main processes lead to liver fibrosis. First, HSCs become activated, resulting in increased cellular proliferation and biotransformation to an activated myofibroblast-like cell. Second, there is an increase in ECM protein synthesis and deposition, predominantly type Ⅰ collagen. TGF-β1 signaling pathway plays a central role in this process, as it is the main stimulating factor for profibrotic ECM synthesis[72].

Although it has been known for some time that vitamin D has anti-proliferative and anti-fibrotic properties and plays an important role in the regulation of ECM, little has been known until recently about the effects of vitamin D on HSCs. Indeed, the finding of a robust VDR expression in HSCs[24] has led to the discovery that VDR signaling can suppress fibrotic gene expression and inhibit proliferation of HSCs[30,73]. Elegant mechanistic studies with VDR knockout mice confirmed the development of spontaneous liver injury with fibrosis in these mice[30]. Moreover, in an experimental mouse model of liver injury, co-treatment with VDR ligand attenuated the progression of liver fibrosis[30]. Consistent with the above studies are the findings of another *in-vitro* study, this time in human HSCs, in which treatment with $1,25(OH)_2D$ caused inhibition of fibrogenesis by inhibiting type 1 collagen formation[74]. The underlying mechanism by which VDR prevents this pathological process has only been recently elucidated and involves the antagonistic action of VDR on SMAD transcription factor - a key transcription factor in the pro-fibrotic process linking TGF-β1[30].

VITAMIN D AS A TREATMENT FOR NAFLD/NASH

In total, the evidence to date suggests that vitamin D may be beneficial in preventing the progression of NAFLD. Therefore, clinical trials that directly evaluate the effect of vitamin D supplementation on disease progression in NAFLD subjects are warranted. Only recently were the results of a small double-blind, placebo-control study in NAFLD subjects published. In this study[75], the investigators randomly assigned NAFLD participants in the vitamin D group (50000 IU every 14 d for 4 mo) and the control group (no vitamin D supplementation). After a follow-up period of 4 mo, there were no significant differences in the serum levels of liver enzymes, HOMA-IR or grades of hepatic steatosis, measured by ultrasound, between the two groups. However there was a significant decrease in the levels of hsCRP and MDA (malondialdehyde) - a marker of lipid peroxidation - in the subjects treated with vitamin D. The negative results of this study on the markers of steatosis and liver injury have to be interpreted carefully given the limited number of participants (n = 53) and the relatively short term of follow up. On the contrary, the confirmation that vitamin D treatment resulted in improvement of inflammatory biomarkers in NAFLD subjects suggests that vitamin D supplementation may be considered as an adjunctive therapy to attenuate systemic inflammation in NAFLD.

CONCLUSION

Vitamin D deficiency is commonly associated with NAFLD and has even been correlated with disease severity. The metabolic, anti-inflammatory and anti-fibrotic properties of vitamin D provide plausible mechanisms by which vitamin D may impact on the various steps of disease progression and severity. Cumulatively, this would suggest that vitamin D replacement might be effective in the treatment of NAFLD. However, controversies exist in the field given the limited number of prospective randomized studies in humans examining the role of vitamin D supplementation in NAFLD, the presence of variability on the methodologies used for detecting vitamin D levels[76] as well as the lack of consensus in the scientific community on defining the optimal levels of vitamin D (> 20 ng/mL *vs* > 30 ng/mL)[77,78].

In conclusion, larger, randomized, placebo-controlled trials are required to better evaluate the efficacy of vitamin D replacement and parameters of therapy in NAFLD. Until then, it is premature to recommend vitamin D supplementation for the specific treatment of NAFLD even though its role seems to be promising.

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REVIEW

Biological therapy in inflammatory bowel diseases: Access in Central and Eastern Europe

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Abstract

Biological drugs opened up new horizons in the management of inflammatory bowel diseases (IBD). This study focuses on access to biological therapy in IBD patients across 9 selected Central and Eastern European (CEE) countries, namely Bulgaria, the Czech Republic, Estonia, Hungary, Latvia, Lithuania, Poland, Romania and Slovakia. Literature data on the epidemiology and disease burden of IBD in CEE countries was systematically reviewed. Moreover, we provide an estimation on prevalence of IBD as well as biological treatment rates. In all countries with the exception of Romania, lower biological treatment rates were observed in ulcerative colitis (UC) compared to Crohn's disease despite the higher prevalence of UC. Great heterogeneity (up to 96-fold) was found in access to biologicals across the CEE countries. Poland, Bulgaria, Romania and the Baltic States are lagging behind Hungary, Slovakia and the Czech Republic in their access to biologicals. Variations of reimbursement policy may be one of the factors explaining the differences to a certain extent in Bulgaria, Latvia, Lithuania, and Poland, but association with other possible determinants (differences in prevalence and incidence, price of biologicals, total expenditure on health, geographical access, and cost-effectiveness results) was not proven. We assume, nevertheless, that health

deterioration linked to IBD might be valued differently against other systemic inflammatory conditions in distinct countries and which may contribute to the immense diversity in the utilization of biological drugs for IBD. In conclusion, access to biologicals varies widely among CEE countries and this difference cannot be explained by epidemiological factors, drug prices or total health expenditure. Changes in reimbursement policy could contribute to better access to biologicals in some countries.

Key words: Inflammatory bowel diseases; Ulcerative colitis; Biological therapy; Access; Europe, Central and Eastern; Crohn's disease

Core tip: Great heterogeneity ranging up to 96-fold difference in access of inflammatory bowel diseases (IBD) patients to biologicals can be found across Central and Eastern Europe (CEE): Poland, Bulgaria, Romania, and the Baltic States have, to date, fallen behind Hungary, Slovakia and the Czech Republic. The following factors did not explain the considerable variations among the CEE countries: differences in prevalence and incidence, price of biologicals, total expenditure on health, geographical access, clinical guidelines, and cost-effectiveness results. We assume that health deterioration linked to IBD might be valued differently against other systemic inflammatory conditions in distinct countries which contributes to the great heterogeneity.

Rencz F, Péntek M, Bortlik M, Zagorowicz E, Hlavaty T, Śliwczyński A, Diculescu MM, Kupcinskas L, Gecse KB, Gulácsi L, Lakatos PL. Biological therapy in inflammatory bowel diseases: access in Central and Eastern Europe. *World J Gastroenterol* 2015; 21(6): 1728-1737 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i6/1728.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i6.1728

INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are idiopathic, chronic inflammatory disorders of the gastrointestinal tract known as inflammatory bowel diseases (IBD). In general, IBD is characterised by flare-ups and remissions of varying duration and severity, and only a minority of patients experience a chronic, continuous disease course[1]. CD may involve any part of the digestive tract, but mainly affects the distal ileum and the colon, whereas UC usually starts in the rectum and extends in a continuous retrograde manner through part of, or the entire colon[1,2]. Approximately 80% of CD patients will require at least one intestinal surgery, while 10%-30% of UC patients will undergo colectomy during their lifetime[1]. Due to early onset, fluctuating disease course, unpredictable prognosis and lack of a cure, IBD poses a considerable burden on patients.

Introduction of biological drugs in the treatment of IBD has brought a paradigm shift in patient management and treatment goals that promoted corticosteroid-free clinical, endoscopic, and biomarker remission[3,4]. Infliximab was the first biological approved by European Medicines Agency (EMA) for treatment of CD in 1999, then 7 years later in UC, adalimumab was registered in 2007 in CD and 5 years later in UC. Furthermore, golimumab received authorisation for the treatment of UC in 2013. Although biologicals have been marketed in Western Europe for over 15 years now, the access is fairly difficult in certain CEE countries. Of note, in 2013, biosimilar infliximab has been approved for the same indications as the original drug and has now been marketed first in the CEE region[5], and may affect the access to biologicals worldwide as well as in the CEE countries.

This study aimed to explore access to biological therapy of IBD patients in nine Central and Eastern European (CEE) countries, namely Bulgaria, the Czech Republic, Estonia, Hungary, Latvia, Lithuania, Poland, Romania and Slovakia. Literature data was systematically reviewed on the epidemiology and disease burden of IBD in these CEE countries. We also aimed to explore whether the access to biologicals is different in these countries and furthermore, to identify possible factors that predispose to regional differences.

EPIDEMIOLOGY

Recent data indicate that the incidence and prevalence of IBD are increasing over time and in different geographical locations[6]. In Europe, the annual incidence of CD and UC ranges between 0.3-12.7 and 0.6-24.3 per 100000 person-years, respectively[6]. European prevalence rates vary between 4.9-505 per 100000 persons for UC and 0.6-322 per 100000 persons in CD[6]. The peak ages for CD and UC occurrence are 20-30 years and 30-40 years, respectively; and paediatric IBD accounts for 7%-20% of all cases[1].

To provide an insight into the epidemiology of IBD in the CEE countries, we relied on the summary introduced by Lakatos *et al*[7] in 2006, and incorporated results of a complementary systematic literature search for the period between 2006 and June 30th, 2014. We included publications that dealt with the 9 selected CEE countries, and excluded those that investigated only paediatric IBD (Table 1).

Overall 17 studies from 7 CEE countries were identified with observation periods varying from 1951 to 2013. No data was available on the epidemiology of IBD from Bulgaria or Latvia. To date, only one multicountry study has been carried out that involved 5 out of the 9 countries of interest[8]. Among CEE countries, the highest incidence and prevalence rates were noted

Table 1 Incidence and prevalence of Crohn's disease and ulcerative colitis in the total population in 9 selected Central and Eastern European countries

Ref.	Country, region/city	Study period	Incidence/10^5		Prevalence/10^5	
			CD	UC	CD	UC
Burisch *et al*[8], 2014[1]	Czech Republic, Prague	2010	5.50	5.5	-	-
	South Bohemia		3.80	3.8		
Bitter and Hulec[45], 1980	Czech Republic, North Bohemia	1968-1978	-	1.3	-	17.6
Nedbal *et al*[46], 1967	Czech Republic	1960-1965	-	1.4	-	10.7
Salupere[47], 2001	Estonia, Tartu County	1993-1998	1.40	1.7	-	-
Kull *et al*[48], 1998	Estonia, Tartu County	1973-1992	0.27	1.5	-	-
Burisch *et al*[8], 2014[1]	Estonia, Southern Estonia	2010	5.20	5.2	-	-
Nagy *et al*[49], 2004	Hungary, Borsod-Abaúj-Zemplén County	1962-1992	-	1.4	-	10.4
Lakatos *et al*[10], 2011	Hungary, Western Hungary	2002-2006	8.87	11.9	115.3	211.1
Lakatos *et al*[9], 2004	Hungary, Veszprém County	I: 1997-2001 P: 1991-2001	2.23	5.89	52.9	142.6
Burisch *et al*[8], 2014[1]	Hungary, Veszprém County	2010	11.5	10.3	-	-
Zvirbliene *et al*[50], 2003[2]	Lithuania	1995-2001	-	-	10.0	30-40
Kiudelis *et al*[51], 2012	Lithuania, Kaunas	2007-2009	1.21	6.56	-	-
Burisch *et al*[8], 2014[1]	Lithuania, Kaunas	2010	2.40	6.1	-	-
Chojecki[52], 1964	Poland, Warsaw	1951-1960	0.66	-	66.0	-
Wiercinska-Drapalo *et al*[53], 2005	Poland, Bialystok	1990-2003	0.10	1.8	-	-
Gheorghe *et al*[54], 1997	Romania, Bucharest	1990-1997	0.42	-	-	-
Gheorghe *et al*[11], 2004	Romania, nationwide	I: 2002-2003 P: 2004	0.50	0.97	1.51	2.42
Toader[55], 2008	Romania, North-East region	1988-2007	1.54	0.35	-	-
Burisch *et al*[8], 2014[1]	Romania, Timis	2010	1.70	2.4	-	-
Príkazská *et al*[56], 1996	Slovakia, nationwide	1994	6.75	-	-	-
Príkazka *et al*[57], 1998	Slovakia, nationwide	1994	-	-	6.75	-
Gregus *et al*[12], 2014	Slovakia	2013	4.60	6.8	80.5	150.5

[1]Aged ⩾ 15 years; [2]Extrapolation by Lakatos *et al*[7], 2006. I: Incidence; P: Prevalence; CD: Crohn's disease; UC: Ulcerative colitis.

Table 2 Estimated number of newly diagnosed and prevalent Crohn's disease and ulcerative colitis patients aged ⩾ 15 years in 9 selected Central and Eastern European countries, 2013

	Number of new patients			Total patient number		
	CD	UC	Total	CD	UC	Total
Bulgaria	208	290	497	6162	11381	17543
Czech Republic	416	416	833	8768	16192	24960
Estonia	58	58	116	1090	2013	3103
Hungary	975	873	1848	9775	17897	27672
Latvia	57	80	137	1695	3131	4826
Lithuania	61	155	216	2482	4584	7066
Poland	1080	1506	2586	32049	59188	91237
Romania	287	405	692	16526	30520	47046
Slovakia	211	311	522	3687	6893	10580
Total	3353	4094	7447	82235	151798	234033

Data sources: Numbers of new patients were estimated based on incidence data by Burisch *et al*[8], 2014. In case of the Czech Republic mean of two available regional incidence data was calculated ($4.65/10^5$ both for CD and UC), and for Bulgaria and Lithuania mean incidence rate of 8 CEE countries (Croatia, Czech Republic, Estonia, Hungary, Lithuania, Moldova, Romania, and Russia) calculated by Burisch *et al*[8] was applied (CD $3.3/10^5$, UC $4.6/10^5$). Total patient number was estimated using data from Gregus *et al*[12], 2014 for Slovakia (CD $80.5/10^5$, UC $150.5/10^5$) and Lakatos *et al*[10], 2011 for Hungary (CD $115.3/10^5$, UC $211.1/10^5$). For the other countries mean prevalence rates of Slovakia and Hungary were applied (CD $97.9/10^5$, UC $180.8/10^5$). Population data were obtained from Eurostat Statistics Database[34]. CD: Crohn's disease; UC: Ulcerative colitis.

in Hungary (incidence CD $8.87/10^5$, UC $11.9/10^5$ and prevalence CD $115.3/10^5$, UC $211.1/10^5$), while the lowest in Romania (incidence CD $0.5/10^5$, UC $0.97/10^5$ and prevalence CD $1.51/10^5$, UC $2.42/10^5$)[9-11]. Nevertheless, comparison of these studies is hampered by the different study designs, investigation periods, length of follow-up, country regions, genetic and lifestyle characteristics, and age-groups included.

CEE was previously seen as a low incidence area. Nonetheless, more recent data has confirmed increasing incidence and prevalence trends. For instance, latest studies highlighted that incidence and prevalence in certain CEE countries, *e.g.*, the Czech Republic, Estonia, Hungary, and Slovakia emerging to that observed in Western and Northern European countries[8,10,12].

The estimated number of patients annually diagnosed with IBD (aged ⩾ 15 years) approached 7500 (55% UC) within the region. Our findings suggest that in 2013, there were approximately 235000 IBD patients (aged ⩾ 15 years) between these countries and the proportion of patients with UC added up to 65% (Table 2). Of note, these patient numbers are extrapolations based on available epidemiology data.

DISEASE BURDEN

IBD is a disabling condition that considerably reduces patients' health-related quality of life and influences their professional, social and personal lifestyle[13]. Due to the early onset and chronic character of the disease, patients have to deal with their disorder throughout their lifetime. The overall mortality of IBD

patients is slightly, but significantly higher than in the general population with standardized mortality ratios of 1.39 for CD and 1.19 for UC, respectively[14,15].

In Western countries, IBD is associated with an excessive economic burden. In 2006, the average direct medical costs of CD amounted to €2898-6960/patient/year in Western Europe[16]. Mean annual per patient direct medical costs of UC ranged from €8949 to €10395, and total economic burden of UC accounted for €12.5-29.1 billion annually in Europe (2008 prices)[17]. Earlier studies from the past decade pointed out that primary cost drivers of IBD were surgical treatments and hospitalizations[18,19]. Nevertheless, recent studies indicated that healthcare costs of IBD have shifted from hospitalization and surgery towards drug therapy, mainly due to the increasing use of biological drugs[20,21]. Besides medical costs, a substantial proportion of patients are young adults and thus, indirect costs related to productivity loss at work account for about 16%-69% of the total burden[17,19-21].

Limited data are available on the costs of IBD from the CEE countries[22-25]. In a recently published paper within the framework of the Epidemiological Committee of European Crohn's and Colitis Organisation (ECCO-EpICom), costs for the first year of follow-up of newly diagnosed patients, including diagnostics and treatment, were estimated[22]. In the CEE region (Croatia, the Czech Republic, Estonia, Hungary, Lithuania, Moldova, Romania, and Russia) annual per patient costs of CD comprised of the following items: diagnostics €1264, surgery €19586, standard treatment €324, and biologicals €9607, respectively, whereas those for UC were: diagnostics €740, surgery €14014, standard treatment €513, and biologicals €1729, respectively. Cost calculation was based on the Danish diagnosis-related group (DRG) financing system and costs of medications were encountered in Danish prices for all countries; thus, results should be interpreted with caution in the CEE[22].

Direct healthcare costs attributable to IBD were investigated in Poland by Meder *et al*[23]. Between 2004 and 2007 medical costs of an acute exacerbation and a 12-mo follow-up period were calculated in 41 IBD patients, of whom 7 received surgical and 3 biological therapy. The average annual per patient costs of treatment amounted to €2968 in CD and €2540 in UC (EUR 1 = PLN 4.142). The bulk of direct costs were related to biological therapy and surgical treatment with mean annual per patient costs of €1565 and €692, respectively[23].

In a multicentre study from Poland, indirect costs in 256 CD patients (aged 18-65 years, biological treatment rate not reported) were determined by a human capital approach (HCA)[24]. Per patient mean annual costs attributable to absenteeism and presenteeism were €2348 and €3011, respectively (EUR 1 = USD 1.344, year 2012)[24].

Recently, Mandel *et al*[25] conducted a research on the indirect costs of IBD among 443 patients in Hungary. Applying the HCA method, average total annual per patient productivity loss was €1880, of which €1450 and €430 incurred due to disability-related productivity loss and sick leaves from work, respectively (EUR 1 = HUF 300, year 2013). Annual per patient costs of presenteeism in CD and UC patients were reported €2605 and €2410, respectively[25].

ACCESS TO BIOLOGICAL THERAPY

So far, the following three biologicals have been registered for the treatment of IBD by EMA: adalimumab and infliximab for the treatment of CD; adalimumab, infliximab, and golimumab for the treatment of UC.

Numbers of gastroenterology centres entitled to administer biological therapy in the CEE countries are presented in Table 3. In the 9 selected countries, on average 784000 inhabitants are covered by a centre; nevertheless, in Romania and Latvia this exceeds the 2 million inhabitants per centre threshold, whereas in Estonia, Slovakia and the Czech Republic fewer than 500000 inhabitants are referred to each centre on average (Figure 1). We found a strong inverse correlation between the number of inhabitants covered by a centre and countries' total expenditure on health (r = -0.83, P = 0.005).

Due to the lack of IBD registries covering the entire patient population in the CEE countries, partial data on biological exposure are available *via* multiple sources such as health insurance databases, IMS sales statistics, ministries of health, national gastroenterology societies, and personal communication (Table 3). We provide an approximate estimation on biological treatment rates estimated from prevalence data of Table 2 and number of patients with biological therapy in Table 3: Hungary 19.1%, Slovakia 18.7%, the Czech Republic 11.3%, Estonia 3.9%, Lithuania 2.9%, Poland 2.8%, Romania 1.5%, Bulgaria 0.7% and Latvia 0.2%, respectively. Rates of UC patients treated with biologicals are as follows: Slovakia 6.4%, Hungary 3.5%, Romania 2.1%, Estonia 1.3%, Lithuania 1%, Bulgaria and Latvia 0%-0%, respectively. Taking into consideration the uncertainty in prevalence data, we also calculated the biological treatment rates based on the number of inhabitants for each country. (This approach disregards the differences in prevalence across the 9 countries.) Biological exposure rates are confirmed by the average number of patients treated with biologicals per 10^5 inhabitants that shows similar distribution (Figure 2). However, these geographical access estimations need to be interpreted with caution since only patients aged $\geqslant$ 15 years were taken into consideration, and number of patients on biologicals aged < 15 is unknown.

Table 3 Number of Crohn's disease and ulcerative colitis patients treated with biologicals and centres where biologicals are administered in 9 selected Central and Eastern European countries, 2014

Country	Number of patients								Centres
	CD[1]			UC[1]				Total	
	Infliximab	Adalimumab	Total	Infliximab	Adalimumab	Golimumab	Total		
Bulgaria	NR	46	46	NR	0	NR	0	46	4
Czech Republic	750	240	990	412	NA	NA	412	1402	26
Estonia	29	13	42	21	5	1	27	69	4
Hungary	970	900	1870	460	170	0	630	2500	16
Latvia	1	2	3	0	0	0	0	3	1
Lithuania	30	43	73	15	31	0	46	119	4
Poland	506	382	888	NA	NA	NA	NA	888	50[2]
Romania	114	139	253	73	540	37	650	903	7
Slovakia	350	340	690	320	110	10	440	1130	13[3]
Total	2750	2105	4855	1301	856	48	2205	7060	125

[1]Including paediatric and adult patients; [2]Approximately; [3]10 adult and 3 paediatric. National gastroenterology societies, ministries of health, IMS data, personal communication. NA: Not available; NR: Not reimbursed; CD: Crohn's disease; UC: Ulcerative colitis.

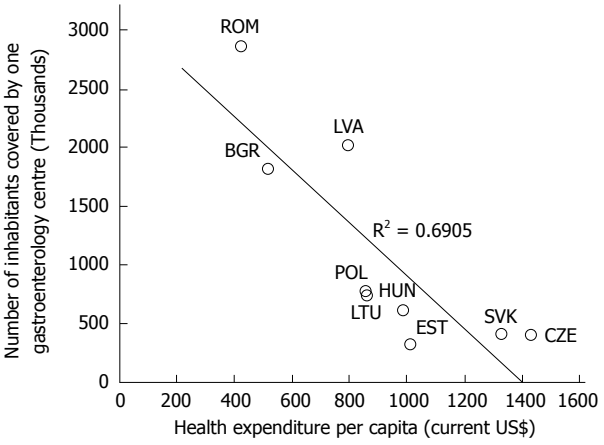

Figure 1 Number of inhabitants covered by one gastroenterology centre entitled to administer biological therapy in 9 selected Central and Eastern European countries, 2014. Population data: Eurostat Statistics Database (2013)[34], total health expenditure per capita (2012): World Bank Databank[26]. BGR: Bulgaria; CZE: Czech Republic; EST: Estonia; HUN: Hungary; LVA: Latvia; LTU: Lithuania; POL: Poland; ROM: Romania; SVK: Slovakia.

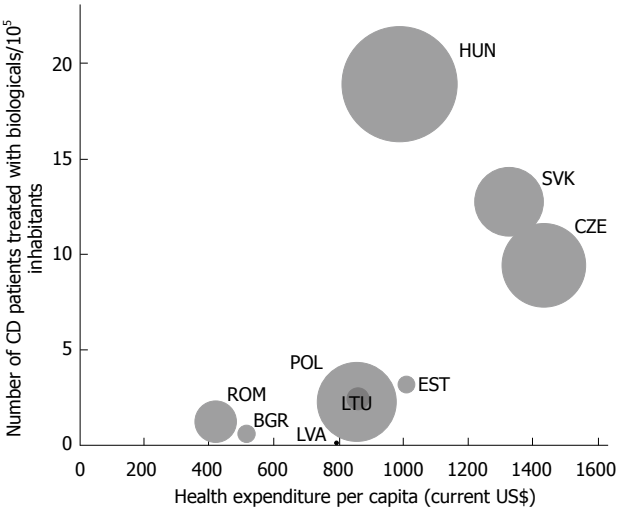

Figure 2 Average number of Crohn's disease patients treated with biologicals per 10^5 inhabitants compared to countries per capita total expenditure on health. Ulcerative colitis would display a similar figure. Sizes of bubbles refer to the absolute number of patients treated with biologicals in each country. Data sources: patient numbers: IMS data (2014 or latest available), population data: Eurostat Statistics Database (2013)[34], total health expenditure per capita (2012): World Bank Databank[26]. BGR: Bulgaria; CZE: Czech Republic; EST: Estonia; HUN: Hungary; LVA: Latvia; LTU: Lithuania; POL: Poland; ROM: Romania; SVK: Slovakia.

Price and reimbursement

To focus on prices of biologicals, some differences can be noted within the CEE region: adalimumab €957-€1262, infliximab €481-€617, and golimumab €1067-€1646 (per dose national list prices)[5].

In most CEE countries, biologicals are covered at 100% by the health insurance system, although share of coverage between pharmaceutical companies and insurance funds occurs in certain countries. For instance, in Bulgaria 25% is paid by the pharmaceutical companies and 75% by the National Health Insurance Fund. Among the Baltic States, biological therapy is compensated by 100% in Lithuania and Estonia, but only 50% of medication cost is reimbursed in Latvia, where the other half is financed by patient co-payment. All three biologicals approved by EMA in IBD indication are reimbursed in CEE except for Bulgaria, where original infliximab and golimumab do not have reimbursement coverage.

From 2014, biosimilar infliximab began to be marketed in the CEE countries resulting in a price reduction of approximately 20%-25%. In Hungary, since May 15, newly initiated biological therapy with infliximab must be undertaken with a biosimilar antibody. A mandatory switch is not recommended; however, relapsers should only be treated with a biosimilar if more than a year has passed since the termination of the previous biological therapy. A somewhat different regulation is applied in Poland, where new patients have to be treated with a biosimilar, and even patients receiving the original drug are forced to switch to biosimilar

infliximab as maintenance therapy. By contrast, in Romania, switch is not mandated, although in order to ensure a price level comparable to the biosimilars for patients, surplus costs generated by the prohibition of substitution are paid by pharmaceutical companies. In Lithuania, from August 1, biosimilar infliximab has to be the first-choice for all newly initiated biological therapies; however, the original antibodies are financed for patients on maintenance therapy with infliximab or adalimumab, and a switch is not allowed. The situation is unique in Bulgaria, where infliximab has not been reimbursed to date, and, hence, IBD patients skipping the original infliximab commence their first biological therapy with a biosimilar. On the other hand, in the Czech Republic either the originally released anti-TNF agents or the biosimilars can be used according to a physician's decision, and moreover, after the introduction of the biosimilars, prices of both the originally released and the biosimilar drug are required by law to be reduced by at least 15%.

Total per capita expenditure on health in the 9 CEE countries varied between $420 (Romania) and $1432 (Czech Republic) (year 2012)[26]. We observed no significant correlation between the average number of patients treated with biologicals per 10^5 inhabitants and total health expenditure (Figure 2). Despite Hungary, Poland, Lithuania, and Latvia having similar total expenditure on health, a higher proportion of patients per 10^5 inhabitants was treated with biologicals in Hungary than in the other three countries. Furthermore, in Slovakia and the Czech Republic a lower proportion of patients per 10^5 inhabitants received biologicals compared to their relatively high total health expenditure.

Eligibility criteria

Based on the current diagnostic and treatment recommendations of ECCO[27,28], national gastroenterology societies have established their own guidelines. Several variations can be found across the CEE countries regarding the clinical criteria defined for eligibility to be treated with biologicals and in financing restrictions; we try to point out some notable differences between those countries, where criteria are clearly stated.

In most countries, moderate to severe luminal CD (Crohn's Disease Activity Index - CDAI > 300 in adults), or perianal or fistulising CD, or moderate to severe UC patients with immunosuppressant or corticosteroid refractory disease, or those with intolerance or contraindication to conventional therapies are eligible to be treated with biologicals. Efficacy of the induction therapy should be evaluated between weeks 12 and 16, and maintenance therapy is reimbursed for those who fulfil the response criteria (luminal CD: $\geqslant$ 70 points decrease in CDAI; fistulising CD: $\geqslant$ 50% reduction in the amount of drainage; UC: $\geqslant$ 50% reduction in UCDAI; corticosteroid-resistant UC: 3 points reduction in Mayo score; corticosteroid-dependent UC: corticosteroid-free remission).

In Bulgaria and Poland, CD patients' maintenance therapy is reimbursed only up to 12 mo; however, in Poland re-treatment is covered after 16 wk after the termination of the previous treatment. Criteria are more strict for UC, mainly severe patients are eligible to receive biological therapy, and additionally, treatment duration is also limited, for example, in Poland only three doses of infliximab without any further continuing treatment can be offered; and in Hungary, where UC patients' maintenance therapy is limited to 12 mo; nevertheless, during later flare-ups, retreatment is allowed.

Besides, different authorisation processes function that can affect the access in CEE. In general, gastroenterologists have to request for the biological drug from the health insurance company at the initiation of the therapy, and additionally they are obliged to report on therapeutic outcomes. During maintenance treatment, prolongation has to be claimed every 6 mo.

DISCUSSION

The objective of this paper is to review the access to biological therapy in IBD across 9 selected CEE countries. The proportion of patients treated with biologicals and average number of patients treated per 10^5 inhabitants were estimated. Potential bias due to the unknown validity of country specific IBD epidemiology was filtered out using this population-based calculation.

In CEE, the estimated proportion of patients treated with biologicals vary from 0.2%-19.1% for CD and from 0%-6.4% for UC. In the United States, a recently published, retrospective analysis of a large database containing pharmacy and medical claims data of almost 1 million IBD patients indicates that 16.8% of CD and 3.5% of UC patients were treated with biologicals (infliximab, adalimumab, certolizumab pegol, natalizumab) between 2010 and 2012[29]. This is similar to the treatment patterns of the best-performing countries from the CEE region.

In England, it is estimated that in CD and UC, 13% and 15% of the clinically eligible patients received biologicals in 2012[30]. Thus, on average 26 CD patients were treated with biologicals (infliximab, adalimumab) per 10^5 adults aged $\geqslant$ 18 years which is higher than the rates observed in any of the CEE countries (Figure 2)[30].

In all countries other than Romania, the lower biological treatment rates were observed in UC compared to CD despite the higher prevalence of UC. A possible explanation for the difference is that the first biological in UC indication was approved in 2007 (8 years after CD); therefore, due to the economic crisis and the subsequently implemented austerity

policies affecting health care spending as well, UC patients in these 9 CEE countries were disadvantaged compared to patients with either CD or with other systemic inflammatory conditions, where biological drugs had been used historically. Also, there are additional, non-economic determinants promoting treatment differences between CD and UC, *e.g.,* higher percentage of UC patients had their disease controlled with "conventional" therapies and the curative surgical option in medical failure[31].

We tried to identify the most important factors that are underlying the differences in biological uptake among the CEE countries. Experts usually state that the following factors might influence the access to biologicals: differences in incidence and prevalence, price of biologicals, per capita total health expenditure, geographical access, clinical and financing guidelines, disease burden, cost-effectiveness results of biologicals, medical professionals' lobbying power, local reimbursement policy, and health care financing mechanisms.

In CEE, access to biologicals is highly diverse, in certain countries such as Hungary, Slovakia, and the Czech Republic, higher number of patients per 10^5 inhabitants are treated with biologicals, whereas in the Baltic States, Poland, Romania, and Bulgaria access to biologicals is rather limited. In addition to IBD, heterogeneous access to biologicals was reported from 6 CEE countries (Bulgaria, the Czech Republic, Hungary, Poland, Romania, and Slovakia) in other inflammatory conditions such as rheumatic diseases[32]. Nevertheless, access rates in IBD vary more extensively across these six CEE countries. Compared to approximately an 8-fold difference noted in rheumatoid arthritis (Poland: 1.3% and Slovakia: 10%)[32], we found up to 27-fold difference in CD (Bulgaria: 0.7%, Hungary: 19.1%). In addition, when considering all the 9 countries, that difference was as high as 96-fold.

Unfortunately, there are a lack of registries on IBD patients on biologicals, and up-to-date epidemiology are missing from some countries (Table 1). We presume, however, that variance in the incidence and prevalence of IBD des not explain such great differences in the access to biological therapy among these 9 countries. It should be addressed that establishing registries would allow not only follow up of patients, and provide valid and reliable data about access rates, but also might favourably enhance financing and reimbursement decision making concerning biologicals and additionally biosimilars.

The difference in the prices of infliximab, adalimumab, and golimumab in CEE[33] does not explain the extent of heterogeneity for their access. Regarding the economic performance, the per capita gross domestic product (GDP) as a percentage of EU-27 countries' ranges from 52.8% (Bulgaria) to 79.6% (the Czech Republic) resulting also a significant differences in total expenditure on health[34]. As an example, the Czech Republic spends 70% more on health compared to Romania and this might contribute to its 8-fold higher access rate. However, comparison of Hungary and Poland which have very similar total health expenditure refutes this assumption since in Poland the exposure to biologicals is approximately 10-fold lower compared to Hungary (Figure 2).

The number and geographic distribution of gastroenterology centres offering biological therapy can also affect the access in some countries. Nevertheless, Figures 1 and 2 indicate, that contrary to a comparable number of patients covered by a gastroenterology centre, Poland and Lithuania lag behind Hungary in terms of biological treatment rates.

Various reimbursement coverage of biologicals is possibly responsible for the diverse access rates in CEE. In all countries but Latvia (50% co-payment), biologicals are fully covered and do not require a co-payment. However, in Romania and Bulgaria, insurance funds and pharmaceuticals share the financing in a defined proportion. All countries apply eligibility criteria based on the ECCO guidelines as a standard for reimbursement, yet there can be marked variations among the countries in terms of severity of disease required and duration of reimbursed maintenance therapy. For example, in Bulgaria and Poland, the duration of maintenance treatment in CD and in Hungary for UC are limited to 12 mo. These obstacles likely contribute to the low access rates found in Poland and Bulgaria but not in Hungary, where despite the 12-mo stopping rule in UC, the highest number of UC patients per 10^5 are treated with biologicals among the CEE countries.

Access to medications is largely determined by healthcare financing mechanisms. Most of the 9 countries share a similar policy and biologicals are covered under itemized financing; therefore, differences in biological uptake are not explainable by this factor. Additionally, in Hungary, a financing guideline on biological drugs draws up patient eligibility criteria. There is a unique situation in Lithuania, where a quota system was established based on the number of patients registered by treating centre, and only one in every four clinical centres could gain quotas to initiate new biological treatments. Thus, from August 1, 2014, a total of 23 new IBD patients will receive biological therapy within the next 12 mo in the whole country.

Most CEE countries have implemented a similar health technology assessment (HTA) based decision-making for reimbursement[35]. It is unlikely that IBD is unfavourably distinguished in countries with established HTA, where reimbursement decisions require cost-effectiveness data[35]. Neither variations of the estimated utility gain achievable until remission as a result of biological therapy (CD: 0.06-0.43, UC: 0.25-0.47) nor cost-effectiveness of biologicals can explain this access gap found between CD and UC in CEE[36-39]. Utilities gained as a result of a therapy are used to generate quality-adjusted life years (QALYs).

QALY is a widely used outcome measure in cost-effectiveness analysis that takes into account both the length and the quality of life spent in a health state[40]. A single abstract can be found concerning cost-effectiveness of biologicals from the CEE countries. In Poland, Goszczynska *et al*[41] conducted a study on cost-effectiveness of infliximab as an induction therapy in severe active UC. In a 12-mo timeframe an incremental cost-utility ratio for infliximab was estimated at €16896/QALY compared to colectomy that is below the official financing threshold (€24326/QALY) (EUR 1 = PLN 4.142)[41]. Recently, Gulácsi *et al*[33] have estimated the cost-effectiveness of biologicals used in gastroenterology, rheumatology, and dermatology. According to the estimates, in the Czech Republic, Hungary, Poland, and Slovakia, cost-effectiveness results are below the threshold of 3 times per capita GDP/QALY applied in reimbursement decision making in many CEE countries. However, in Bulgaria and Romania under certain conditions this ratio exceeds the threshold[33]. Hence, variations of cost-effectiveness ratios in six out of the 9 CEE countries do not justify the heterogeneity; for example, despite the calculated cost-effectiveness data in Poland, exposure to these drugs is rather low.

Finally, in the field of rheumatology many more patients are treated with biologicals than in IBD across the CEE countries[32]. However, the prevalence of rheumatoid arthritis (RA) remains higher than that of IBD with a prevalence of 610/10^5 inhabitants reported in the Czech Republic and a 0.5% prevalence in Hungary[42,43]. In addition, comparison of utility gain achievable until remission as a result of biological therapy is estimated to be similar to CD (0.06-0.43), UC (0.25-0.47), and RA (0.15-0.40)[36-39,44]. Interpreting these health gain findings requires caution. Possible methodological differences must be considered such as applied outcome measures, patients' baseline quality of life, time frames, and study design. Therefore, health gain differences cannot explain inequalities in access rates between IBD and RA.

CONCLUSION

Access to biologicals varies greatly (up to 96-fold) in the selected CEE countries that raises inequity concerns regarding access to treatment. To date, biological use in IBD in Poland, Bulgaria, Romania, and the Baltic States is much lower compared to Hungary, Slovakia and Czech Republic. The reason for this heterogeneity in the access to biologicals among the CEE countries has not been clarified. Differences in prevalence and incidence of IBD, prices of biologicals, total expenditure on health, geographical access, and cost-effectiveness results does not explain the above variation. Variations of reimbursement policy might explain the differences to a certain extent in Bulgaria, Latvia, Lithuania, and Poland. It may be also hypothesized that health disability linked to IBD might be valued differently against other systemic inflammatory conditions in distinct countries. Further research, however, is needed to better understand the key factors contributing to the above differences and investigating future trends.

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REVIEW

Management of patients with hepatitis B in special populations

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Abstract

The development of effective nucleos(t)ide analogs (NAs) against hepatitis B virus (HBV) has improved the outcome of patients with chronic hepatitis B (CHB). This review updates issues related to the management of CHB patients included in special populations. Entecavir (ETV) and tenofovir (TDF) represent the currently recommended first-line NAs in patients with HBV decompensated cirrhosis. The combination of HBV immunoglobulin (usually for a finite duration) and NA is considered the standard of care for prophylaxis against HBV recurrence after liver transplantation. TDF is the best choice for hemodialysis patients and in patients with chronic kidney disease with nucleoside resistance. ETV and telbivudine are the preferred options in naïve renal transplant recipients and with low viremia levels, respectively. All hepatitis B surface antigen (HBsAg)-positive candidates should be treated with NAs before renal transplantation to achieve undetectable HBV DNA at the time of transplantation. Conventional interferon or NAs can also be used in children, on the basis of well-established therapeutic indication. Pregnant women at high risk of perinatal transmission could be treated with lamivudine, telbivudine or TDF in the last trimester of pregnancy. HBsAg-positive patients under immunosuppression should receive NA pre-emptively (regardless of HBV DNA levels) up to 12 mo after its cessation. In HBsAg negative, anti-HBc positive patients under immunosuppression, further studies are needed to form a final conclusion; however, it seems that anti-HBV prophylaxis is justified in such patients with hematological diseases and/or for those receiving rituximab-containing regimens, regardless of their anti-HBs or serum HBV DNA status.

Key words: Hepatitis B; Antiviral therapy; Tenofovir; Entecavir; Telbivudine

Core tip: The management of hepatitis B virus (HBV) infection in special populations is reviewed. HBV patients with decompensated cirrhosis should receive nucleos(t)ides analogs (NAs) before and after liver transplantation. The choice of NA for patients with chronic kidney disease, renal transplant candidates and

recipients depends on viremia levels, the severity of renal dysfunction and previous viral resistance. Children at the immune-active period may receive interferon or NAs. Pregnant women at risk of perinatal transmission should receive class B antiviral drugs or LAM. HBV patients receiving immunosuppressives should receive antiviral therapy based on HBV serological profile, HBV DNA detectability and intensity of immunosuppression.

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INTRODUCTION

More than half a million people with hepatitis B virus (HBV) infection die annually from complications of chronic hepatitis B (CHB), mainly the development of liver decompensation and/or hepatocellular carcinoma (HCC)[1]. Untreated patients with HBV decompensated cirrhosis (HBV-DeCi) have a 5-year survival rate of only 14%-35%[2]. The major breakthrough in the field of therapy of CHB patients is the implementation of oral nucleos(t)ide analogs (NAs). Should they be instituted according to the international guidelines, they will eliminate viral replication and l improve liver dysfunction and survival[2,3]. In fact, the newer NAs [*i.e.,* entecavir (ETV) and tenofovir (TDF)] are potent antiviral agents with a minimal or even nonexistent risk of resistance and, therefore, they represent the currently recommended first-line for the therapy of CHB[3].

In all phase Ⅲ pivotal trials, NA efficacy and safety was assessed in CHB patients recruited with strict inclusion criteria. However, in the "real world" daily clinical practice, there are remain many CHB patients, who, because of their particular characteristics, have been excluded from the registration trials. These CHB patients are generally referred to as special populations. Although, they may be in need of more urgent antiviral treatment, such as those with HBV-DeCi, their therapeutic manipulation is usually based on a relatively low degree of evidence (*e.g.,* expert opinion or non-randomized trials). Consequently the decision as to whether special populations with CHB need treatment or not, which NA suits them best and the need for other specific management options, require careful consideration.

The specific populations with CHB can be divided in several groups and subgroups, determined on various characteristics, such as age, severity of liver disease and events/cormorbidities that may change the natural history of HBV infection. The present review focuses on the most frequently seen special populations with CHB. These are patients with decompensated cirrhosis, liver transplant (LT) recipients; patients with chronic kidney disease (CKD) and renal transplant recipients; patients under immunosuppressive therapy or chemotherapy; and finally young patients and pregnant women.

CHB PATIENTS BEFORE AND AFTER LIVER TRANSPLANTATION

Before liver transplantation

CHB is a dynamic disease that can change over time, resulting in serious decompensation[4]. All patients with HBV-DeCi should be commenced on NAs, regardless of viral load and ALT activity. Several lines of evidence demonstrated that these agents were generally well tolerated in the long-term and they suppressed viral replication, preventing possible flares in disease activity and the occurrence of HCC[5,6]. Such patients could be selected for LT if they present hepatic dysfunction [Child-Pugh score (CTP) $\geqslant$ 7 or model for end stage liver disease (MELD) $\geqslant$ 10] and/or at least one major complication (ascites, variceal bleeding or hepatic encephalopathy)[7,8]. The application of NAs prompted a new era in LT of HBV-DeCi patients, because they reduced the rates of recurrence remarkably and improved their prognosis dramatically (survival rates up to 90% over 5 years after LT)[9]. While awaiting for LT, patients should be followed closely, at least every 3 mo, for virological response and potential virological breakthrough, by applying a sensitive polymerase chain reaction (PCR) assay[3,10]. All data suggest that an effective pretransplant anti-HBV therapy prevents post-transplant HBV recurrence[11]. hepatitis B surface antigen (HBsAg)-positive candidates treated with NAs could maintain undetectable HBV DNA, ameliorate liver function and present long term survival after LT[12-16]. Interestingly, liver function may improve to such an extent that some patients might not need transplantation at the end[17-23] (Table 1). The critical parameters affecting the outcome of patients with HBV-DeCi under antiviral agents have been controversial. The baseline severity of liver disease, expressed by the CTP score or the baseline bilirubin and creatinine levels[24], and the levels of viral load in which antiviral treatment is started, may be potential influencing factors. Antiviral therapy initiation at earlier stages is associated with better liver function recovery (Table 1).

Pretransplant mainstay therapy should be potent, with high-genetic barrier agents (*i.e.,* ETV or TDF monotherapy), which present long-term efficacy, very good virological responses, low resistance rates and result in reduction of liver fibrosis[10,25]. For example, a recent study showed that ETV administration in HBV-DeCi patients had a beneficial impact on mortality[26]: those treated with ETV for 24 wk presented greater reduction in ALT levels and MELD score, compared with those commenced on lamivudine. The critical weak point of ETV and TDF is

Table 1 Studies of nucleos/tide analogs in patients with hepatitis B related decompensated cirrhosis (adapted by Cholongitas *et al*[88])

Ref.	Number of patients	NA(s) used	1-yr data		Prognostic factors for the outcome
			↓ CTP score ⩾ 2, (%)	MELD score ↑	
Fontana *et al*[17]	154	LAM	NR	NR	Serum bilirubin and creatinine levels at baseline
Schiff *et al*[18]	226	ADV	NR	-2	NR
Shim *et al*[19]	70	ETV	49	-2.2	NR
Liaw *et al*[20]	45/45/22	TDF/TDF + FTC/ETV	26/48/42	-2/-2/-2	NR
Chan *et al*[21]	114/114	LdT/LAM	32/39	-1.0/-2.0	NR
Hyun *et al*[22]	45/41	ETV/LAM	NR/NR	-4.9/-3.7	Baseline CTP and MELD at 3 mo
Cholongitas *et al*[23]	52	ETV/TDF	23.8/19.3	-0.4/-2.2	Changes in MELD score between baseline and 6 mo

NA: Nucleos/tide analogs; ADV: Adefovir; CTP: Child-Turcotte-Pugh; ETV: Entecavir; TDF: Tenofovir; FTC: Emtricitabine; HBV: Hepatitis B virus; LAM: Lamivudine; LdT: Telbivudine; MELD: Model for end stage liver disease; NR: Not reported.

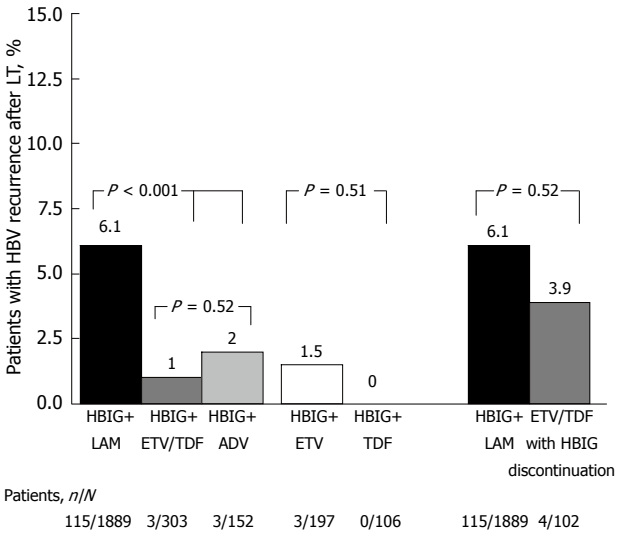

Figure 1 Risk of recurrent hepatitis B virus infection after liver transplantation in relation to the type of post-transplant hepatitis B virus prophylaxis[34,35]. HBIG: Hepatitis B immunoglobulin; LAM: Lamivudine; ETV: Entecavir; TDF: Tenofovir; ADV: Adefovir.

their higher cost compared with lamivudine and the potential TDF nephrotoxicity[27], although the latter was not confirmed in a recent randomized trial[20]. Similarly, lamivudine and telbivudine are limited by drug resistance, and adefovir is limited by its high cost, lower potency and slower onset of action[28]. However, clinicians should be aware that telbivudine can ameliorate creatinine clearance in patients with CHB[29,30] and could be effective in cases of moderate increase of viral load[31,32]. Other therapeutic NA options are the combination of emtricitabine plus TDF; however, this presents a similar efficacy to TDF or ETV monotherapy[20], but at a higher cost. Finally, pre-LT management should include surveillance of lifestyle factors, comorbid conditions and drug interactions[33].

After liver transplantation

In general, management after LT includes prophylactic and therapeutic approaches. Again, lamivudine is not considered an optimal first-line choice because of the elevated rates of viral resistance. In our review[34], the patients treated with HBV immunoglobulin (HBIG) and lamivudine had HBV recurrence more frequently compared with those commenced on HBIG and adefovir. More recently, we showed that the combination of HBIG and ETV or TDF is the best prophylaxis, almost eliminating post-transplant HBV recurrence (< 2%)[35] (Figure 1). Nevertheless, the high cost of HBIG and the fact that more and more patients undergo LT with undetectable HBV-DNA, has encouraged physicians to test either a shorter course of HBIG (with continuation of NA)[36,37], or HBIG-free prophylactic regimens with mono- or dual NA[35,38]. To select the appropriate group of LT recipients in whom HBIG withdrawal, or even no use at all, might be applicable, physicians should be aware of the risk of post-transplant recurrence. HBV DNA ⩾ 20.000 IU/mL and HBeAg positivity at the time of LT are associated with high risk of HBV recurrence, whilst HBV DNA clearance, as well as fulminant HBV and hepatitis D virus coinfection, pose less risk of HBV recurrence[39].

To date, the combination of an NA with a low dose of HBIG[34] is the preferred therapeutic regimen. Another option has been the use of vaccination instead of HBIG[40,41]. Active immunization with two courses of an accelerated schedule of double-dose recombinant HBV vaccine has been applied after LT. However, the results regarding patient response were conflicting, thus further studies are needed to confirm the application of this strategy in clinical practice.

Finally, regarding the use of liver grafts from anti-HBc positive donors, in our systematic review[42], we showed that these grafts can be used safely in HBsAg negative LT recipients. In these cases, anti-HBc/anti-HBs positive recipients may need no prophylaxis at all, while anti-HBc and/or antiHBs negative recipients should receive long-term prophylaxis with lamivudine (Figure 2).

The recurrence of CHB after LT is determined by the redetection of serum HBsAg and/or serum HBV DNA, which is usually connected with biochemical or clinical evidence of active liver disease. The treatment

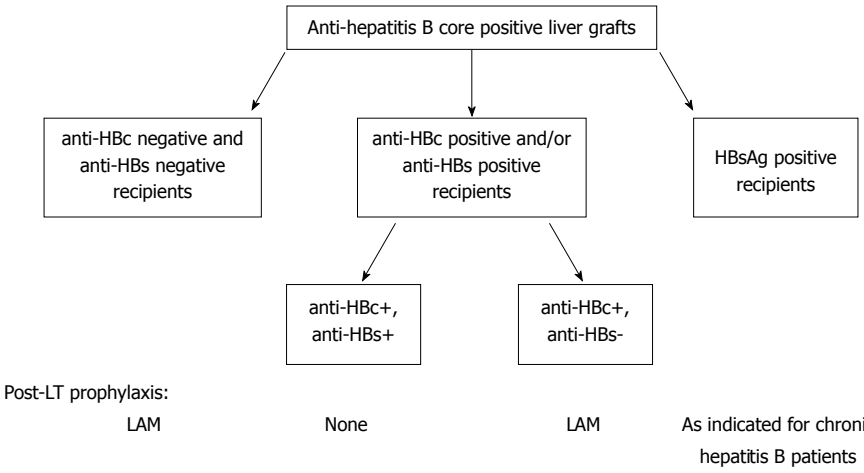

Figure 2 Proposed algorithm for allocation and management of anti-HBc positive liver grafts. Such grafts should be first offered to HBsAg positive, then to anti-HBc and/or anti-HBs positive, and ultimately to HBV naive (both anti-HBc and anti-HBs negative) recipients[42]. LT: Liver transplantation; HBIG: Hepatitis B immunoglobulin; LAM: Lamivudine.

Table 2 Dosage adjustment of nucleos(t)ides analogs in patients with chronic hepatitis B according to the creatinine (CrCl)[44]

CrCl (mL/min)	Lamivudine	Telbivudine	Adefovir	Entecavir[1]	Tenofovir
⩾ 50	100 mg/d	600 mg/d	10 mg/d	0.5 mg/d	245 mg/d
30-49	50 mg/d	600 mg/2nd day	10 mg/2nd day	0.25 mg/d	245 mg/2nd day
10-29	25 mg/d	600 mg/3rd day	10 mg/3rd day	0.15 mg/d	245 mg/3rd-4th day
< 5-10 or HD[2]	10 mg/d	600 mg/3rd-4th day	10 mg/wk	0.5 mg/wk	245 mg/wk[3]

[1]Recommendations only for nucleos(t)ide analog naïve patients (in lamivudine resistance the dosage is double); [2]In patients undergoing HD, all agents should be given once weekly after an HD session; [3]Only for patients on HD. HD: Hemodialysis.

of HBV recurrence depends on the NA that LT recipient was receiving before recurrence. TDF should be administered to patients with prior lamivudine resistance or to those receiving long term ETV[3,43] and the most potent combination of TDF and ETV should be used in patients with multidrug resistant HBV strains.

CHB PATIENTS BEFORE AND AFTER KIDNEY TRANSPLANTATION

Patients with CKD represent a very special population because of impaired immunity of renal failure, the many co-morbidities and the use of multiple medications[44]. They present a heterogeneous patient group, separated into three subgroups: patients with HBV-related nephropathies (membranous/membranoproliferative/IgA glomerulopathy/polyarteritis nodosa)[45-47], patients receiving hemodialysis (HD) and the renal transplant recipients. The course of CHB has a significant impact on the management of all these patient categories and affects their morbidity and mortality[48].

All HBsAg positive patients should undergo baseline renal evaluation, both before the start of antiviral treatment and during its administration. During long-term therapy, minimal rates of creatinine clearance decline have been reported with all NAs, except for telbivudine. Regular renal monitoring ensures prompt diagnosis and management of kidney disease, as well as adjustment of drug doses to renal function or if patients are on regular HD, after each session (Table 2)[3,44,49].

Patients receiving HD are high-risk individuals for CHB, because they are very susceptible to nosocomial transmission and occult HBV infection[50]. The latter might account for the potential risk of transmission during HD service and reactivation of HBV after renal transplantation (RT). Diabetes mellitus increased the possibility of occult HBV infection in patients on HD[51]. Vaccination is an essential component of preventive healthcare measures in this high-risk population, and it should not be underutilized because of poor response[52]. Special vaccination regimens are recommended, including double dose vaccination (40 mg each) in four doses, preferably applied before HD initiation. Serology should be performed every year, and a booster dose should be given if antibody titers are below 10 mIU/mL. Additional parameters complicating the diagnosis and the clinical course of CHB in patients on HD are the minimal or no increase in liver function tests[53], the lower viral load levels, because of its clearance by HD[54] and the high bleeding risk related to clotting disorders and intradialysis anticoagulant therapies. Thus, transjugular

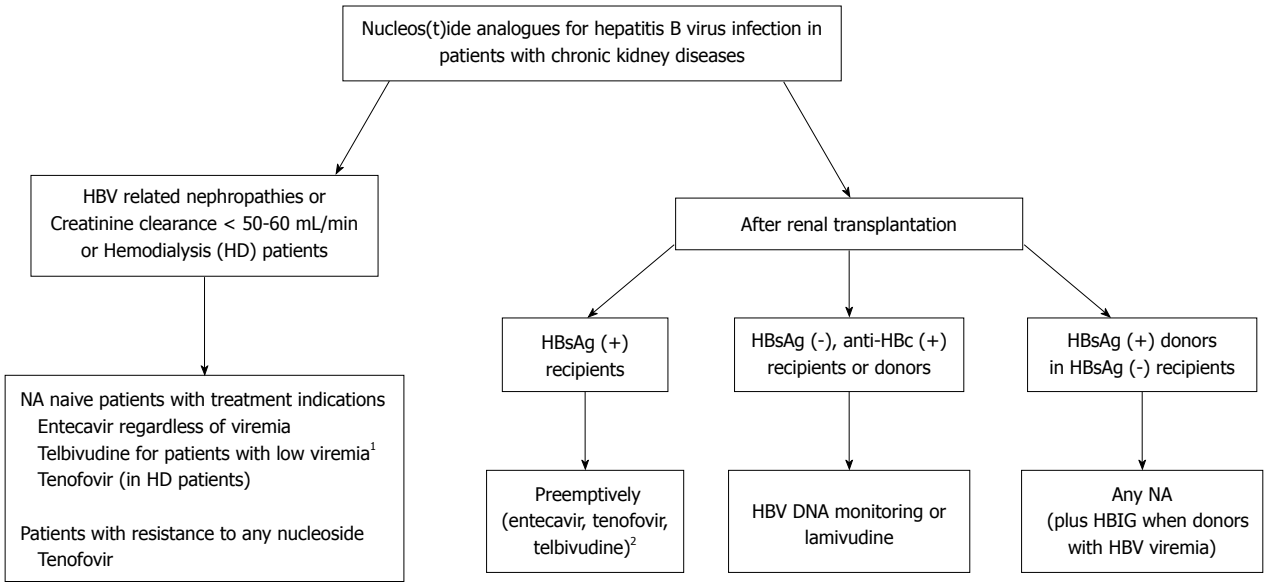

Figure 3 Proposed algorithm for the management of patients with chronic hepatitis B infection and kidney diseases. [1]Low viremia is considered as HBV DNA levels < 10^8 or < 10^6 IU/mL for HBeAg-positive and HBeAg-negative patients, respectively; [2]The choice based on similar criteria as before renal transplantation. NA: Nucleos(t)ide analogs; HBV: Hepatitis B virus; HBIG: Hepatitis B immunoglobulin[58].

liver biopsy in specialized centers and other non-invasive procedures, such as transient elastography, are the preferable option for fibrosis staging[55,56].

Antiviral therapy

With the advent of NAs, interferon (IFN) use has been limited to young patients with HBV-related glomerulopathy without cirrhosis, psychosis or autoimmune disease[57]. IFN has been poorly tolerated by patients with CKD, has shown relatively low efficacy and has set RT recipients under the risk of acute rejection[3], and thus, it is contraindicated.

Patients with HBV-related nephropathies, in which kidney disease is induced *via* the immune-complex, may respond highly to antiviral therapy[6], while those who need immunosuppressive therapy ideally should start antiviral treatment one month before treatment, continued for at least 12 mo after last dose of immunosuppressive drug[6]: ETV regardless of viremia, or telbivudine for patients with low viremia (*i.e.,* HBV DNA levels < 10^8 or < 10^6 IU/mL for HBeAg-positive and HBeAg-negative patients, respectively. However, ETV has low efficacy when there is lamivudine resistance; therefore, TDF should be used in that setting (Figure 3)[58].

Management of HBV patients with CKD requires special manipulation, a multidisciplinary approach and thorough renal monitoring. The administration of NAs has increased the prognosis of patients with CKD dramatically and has prevented the HBV recurrence after RT[3,59]. In patients with CKD and treatment indications for HBV infection, ETV is considered the first choice, regardless of viremia. Telbivudine is the best option when patients present low creatinine clearance and low viremia levels. Telbivudine has been proved efficacious in causing eGFR elevation in CHB patients with high risk of renal impairment[29,30]. TDF is the best choice during lamivudine resistance[58], but concerns exist regarding TDF use, because few cases of ostemalacia and Fanconi syndrome have been documented[60]. Physicians should be vigilant about these side effects and monitor patients closely who are under these medications, especially when creatinine clearance is below 50 mL/min.

Regarding patients on HD, antiviral treatment should be given to those with active or fibrotic liver disease and to renal transplant candidates. In general, RT offers higher survival and better quality of life in HBV positive patients on HD, with the condition that they would be under antiviral prophylaxis, since it is easier to prevent than treat CHB reactivation[61]. If no antiviral prophylaxis is administered after RT, immunosuppressive therapy would predispose the patients to rapidly progressive fibrosing cholestatic hepatitis, even if the underline liver disease was mild before RT[62]. Patients with HBV compensated cirrhosis are precluded from RT because they present high risk of hepatic decompensation after solitary RT, while patients with HBV-DeCi may only undergo combined liver and kidney transplantation[63]. In HD patients, ETV or TDF are considered first line agents, because of their high potency and the high genetic barrier to resistance[3]. Again, TDF is the first choice in lamivudine resistance[3,58] (Figure 3).

All HBsAg-positive RT candidates should be commenced on NAs before RT, regardless of the baseline liver histology and serum HBV DNA level[64]. NAs should be continued after RT to retain viral load clearance and prevent liver decompensation and fibrosis[3]. Oral antiviral treatment raised patient and

graft survival significantly; whereas a decade ago, HBsAg positivity was a significant predisposing factor for high mortality and graft loss[65,66].

The choice of the NA for HBsAg-positive RT is decided on an individual basis, according to the patient's HBV-DNA levels before transplantation and the previous exposure to NA(s). Lamivudine has been used extensively in this setting, but its results have been similar to those in other CHB populations. Thus, ETV, regardless of viremia and creatinine clearance, or telbivudine for patients with low viremia (*i.e.,* HBV DNA levels < 10^8 or < 10^6 IU/mL for HBeAg-positive and HBeAg-negative patients respectively) or TDF for cases with creatinine clearance > 60 mL/min (or history of resistance to lamivudine) could be proposed as the best choices (Figure 3). Although NAs should be continued lifelong after RT, there is a recent study showing safe antiviral withdrawal in four HBV positive RT patients who presented complete suppression of HBV infection having received antivirals for 14.3 mo. They remained negative for HBV DNA for a median 60.5 mo[67].

CHB IN CHILDREN

Most children remain at the immune-tolerant phase of CHB until late childhood or adolescence[68]. This phase is characterized by very high viral load, normal transaminase levels and lack of active disease in liver biopsy[68]. During the immune-tolerant phase, currently available treatments of CHB have no established benefit and should not be administered[68]. However, transaminase levels should be monitored every 6-12 mo in children who are at the immune-tolerant phase, because some will progress to the immune-active phase of CHB[69]. During the immune-active phase, viral load declines, transaminase levels increase and hepatic inflammation with potentially fibrosis develop[69]. According to current guidelines, children presenting HBV DNA levels ⩾ 2000 IU/mL at the immune-active period and persistently elevated alanine transaminase levels > 1.5 times the upper limit of normal [on at least two occasions over at least 6 mo in HBeAg(+) children or on at least three occasions over at least 12 mo in HBeAg(-) children] are potential candidates for treatment[68]. Liver biopsy should be considered at this point as well. Treatment is indicated in case of moderate or severe inflammation or fibrosis[68].

Regarding the choice of CHB treatment, small studies showed that treatment with conventional IFNα for 24 wk accelerates HBeAg clearance and antiHBe seroconversion[70-72]. Improvements in liver histology and increased rates of HBsAg clearance were also reported in IFNα-treated children[71,72]. High transaminase levels, low viral load and greater inflammatory activity in liver biopsy were associated with higher response rates to IFNα in a few studies[70].

Concerning NA(s), a pivotal randomized trial including 288 children with HBeAg(+) CHB, showed that treatment with lamivudine for 52 wk was well tolerated and induced a virological response (HBeAg clearance and undetectable HBV DNA) in 23% compared with 13% of children treated with placebo (P = 0.04)[73]. However, genotypic resistance to lamivudine developed in 19% of children treated with lamivudine at week 52[73]. In a more recent study including 106 adolescents (12-18 years-old) with CHB [91% HBeAg(+)], a 73-wk treatment with TDF resulted in a virological response in 89% of patients compared with 0% in patients treated with placebo (P < 0.001)[74]. ALT normalization occurred in 74% and 31% of patients treated with TDF and placebo respectively (P < 0.001)[74]. However, HBeAg clearance rates did not differ between the two groups[74]. Higher ALT levels and low viral load were associated with higher response rates to TDF treatment. TDF was safe and no patients developed resistance[74].

Current guidelines recommend a conservative management approach and careful treatment evaluation in children with CHB[3]. IFN is the agent of choice, while NAs are a second-line treatment[68]. IFN is approved for use in children ⩾ 1 year-old and is given thrice weekly at a dose of 6 MU/m^2 (maximum of 10 MU) for 6 mo[68]. In contrast, PEGylated IFN is not licensed for use in children with CHB[68]. Lamivudine and ETV are approved for use in children ⩾ 2 years old, adefovir and TDF for adolescents ⩾ 12 years old, whereas telbivudine is approved for adolescents ⩾ 16 years old[10,75]. Lamivudine is administered at a dose of 3mg/kg per day (maximum of 100 mg) once daily and the other NAs at the usual adult doses[10,75]. The optimal duration of treatment with these agents in children remains unknown[68]. Under current circumstances, treatment should be given for at least 6-12 mo after HBeAg seroconversion[75], and indefinitely in patients who do not achieve HBeAg seroconversion[75].

CHB IN PREGNANCY

All pregnant women should be screened for the presence of CHB[10]. CHB positivity does not affect the pregnancy outcome[76] and vice versa: pregnancy does not have an impact on CHB course or activity[77]. However, CHB flares occur in the post-partum period and might lead to HBeAg clearance[77].

IFN, lamivudine, adefovir and ETV are listed by the FDA as pregnancy category C drugs (*i.e.,* animal reproduction studies have shown an adverse effect on the fetus and there are no adequate and well-controlled studies in humans), whereas telbivudine and TDF are pregnancy category B (*i.e.,* animal reproduction studies have failed to demonstrate a risk to the fetus and there are no adequate and well-controlled studies in humans)[3]. Accordingly, the treatment of women of reproductive age contemplating pregnancy and not presenting advanced fibrosis has to be postponed until post delivery[3]. In cases of

advanced fibrosis or cirrhosis, treatment is urgent, with PEGylated IFN representing the first line option, because of its finite treatment duration[3]. In women with no response to IFN or other contraindications, TDF is the treatment of choice, providing that it would be continued during pregnancy[3]. When pregnancy is confirmed in women who are on IFN or NA treatment other than TDF, treatment is discontinued if there is not advanced fibrosis or cirrhosis; if there is, it is continued with the substitution of current medication with TDF[3]. Where medications are withheld during pregnancy, close monitoring is needed because of hepatic flare risk[3].

Whether the NAs use in pregnancy prevents perinatal HBV transmission is an area of uncertainty. In CHB endemic areas perinatal transmission occurs in 70%-90% of children born from HBeAg(+) mothers[78]. It is well established that the risk of progression from HBV infection to CHB is highest (approximately 90%) in infants born from women with CHB compared with patients who are infected with HBV later in life[79,80]. The risk of perinatal HBV transmission is substantially reduced by the combined prophylaxis of HBV immunoglobulin and HBV vaccination[78]; however, it remains high in women with increased viral loads[81,82]. Indeed, perinatal transmission of HBV is observed in approximately 8%-9% of women with high viral loads (> 10^7-10^8 copies/mL), despite infant immunoprophylaxis[81,82]. In a meta-analysis[83] incorporating 15 randomized controlled trials (n = 1693 pregnant women), treatment with lamivudine started at the 28th gestational week was safe and reduced the risk of HBV transmission. However, lamivudine did not show an effect on HBV transmission in women with HBV DNA levels > 10^8 copies/mL[83]. In a more recent, open-label, uncontrolled study[81], treatment with telbivudine started at the 20th to 32nd gestational weeks and was not only safe, but also prevented all cases of HBV transmission in women with HBV DNA levels > 10^7 copies/mL. Interestingly, perinatal HBV transmission occurred in 8% of women treated only with HBV immunoglobulin and HBV vaccination but not telbivudine[81]. Observational studies[84] suggest that treatment with lamivudine or TDF during pregnancy does not increase the risk of major birth defects. Therefore, women with high viral loads (> 10^6 IU/mL) should be treated with lamivudine, telbivudine or TDF in the last trimester of pregnancy to reduce the risk of HBV transmission[3].

CHB PATIENTS UNDER CHEMOTHERAPY OR IMMUNOSUPPRESSION

After HBV exposure, the virus may persist in the liver and other extra-hepatic sites for long periods, posing a risk of reactivation in individuals who receive chemotherapy or immunosuppressive therapy[85]. Although the precise factors associated with risk of reactivation are not well understood[85,86], viral and host factors, as well as immunosuppressive therapy characteristics, are involved[87]. For example, high risk of HBV reactivation is associated with the use of rituximab. The latter is a monoclonal antibody against the CD20 receptor of B lymphocytes[86] and it is used alone or in combination with steroids or other regimens. Currently, rituximab is considered the optimal treatment for B cell lymphomas[88], but its use has been extended in several other hematological and non-hematological diseases.

In clinical practice, any type of immunosuppressive therapy can lead to HBV reactivation, in both HBsAg positive and HBsAg negative/antiHBc positive patients[85]. Thus, it is highly recommended that all candidates for chemotherapy and immunosuppressive therapy should be screened for the HBV (HBsAg and anti-HBc). In HBsAg-positive candidates, NA(s) should be received pre-emptively before immunosuppressive therapy, regardless of baseline HBV DNA levels and for 12 mo after its cessation[85,86]. According to the current guidelines, lamivudine can be used only in HBsAg-positive candidates with low HBV DNA (< 2000 IU/mL) and when a finite and short duration of immunosuppression is scheduled, otherwise the candidates should be protected with a new NA (*i.e.,* ETV or TDF)[3].

Although HBsAg negative/anti-HBc positive patients have significantly lower risk of HBV reactivation compared with HBsAg positive patients, there are many reports of HBV reactivation in these patients, because the prevalence of anti-HBc is higher than that of HBsAg[3]. However, no standard management to prevent HBV reactivation has been established in this setting. In our recent systematic review (unpublished data) including more than 3300 HBsAg negative/anti-HBc positive patients, the rates of HBV reactivation were significantly lower in patients with non-hematological than with hematological diseases (2.5% *vs* 7.8%, $P < 0.001$), as well as in those under rituximab free compared with rituximab-containing regimens (3.5% *vs* 7.9%, $P < 0.001$). Based on these findings, we concluded that anti-viral prophylaxis should be given in HBsAg negative/anti-HBc positive patients with hematological diseases and/or those who are going to receive rituximab-containing regimens, regardless of their anti-HBs and serum HBV DNA status. On the other hand, HBsAg negative/anti-HBc positive patients with non-hematological diseases and/or those who are going to receive rituximab free regimens seem to require anti-HBV prophylaxis only if they have detectable HBV DNA. Nevertheless, further studies are needed to form final conclusions, particularly in specific groups of patients, such as those with solid tumors under chemotherapy. Lamivudine seems to represent an effective option in these cases, and clinicians should

Table 3 Management of chronic hepatitis B in special populations

Special population	Management
Before and after liver transplantation	Before: entecavir or tenofovir (± telbivudine in the presence of renal dysfunction) After: HBIG plus entecavir or tenofovir (consider telbivudine in the presence of renal dysfunction)
Before and after kidney transplantation	Before: entecavir or telbivudine or tenofovir (Figure 3) After: entecavir or telbivudine or tenofovir in HBsAg(+) recipients [plus HBIG when HBsAg(-) recipients receive graft from HBsAg(+) donor with HBV viremia] (Figure 3)
Pregnancy	lamivudine, telbivudine or tenofovir in the last trimester of pregnancy when HBV DNA > 10^6 IU/mL
Children	Interferon or nucleos(t)ide analogue (check age of child)
Under immunosuppressive regimen	HBsAg-positive candidates: lamivudine when baseline HBVDNA < 2000 IU/mL and short period (< 12 mo) of immunosuppression; otherwise: ETV or TDF HBsAg-negative/anti-HBc positive candidates: (1) if baseline HBV-DNA detectable: as HBsAg-positive candidates; (2) otherwise: lamivudine only in hematological diseases or rituximab containing regimens

HBV: Hepatitis B virus; HBIG: Hepatitis B immunoglobulin; HBsAg: Hepatitis B surface antigen; ETV: Entecavir; TDF: Tenofovir.

continue anti-HBV prophylaxis and/or the follow-up of such patients for at least 12 mo after discontinuation of immunosuppression.

CONCLUSION

Significant progress in therapies for HBV infection has led to improvements in the management of CHB patients with decompensated cirrhosis and after LT. The former group should be treated with ETV or TDF, which may lead to stabilization or even improvement of liver disease and possible withdrawal from the waiting list for LT. After LT, the combination of HBIG (at least for a certain period) and ETV or TDF appears to be the most effective approach, while ETV and TDF seem to have no difference in their impact on renal function[36]. HBIG-free prophylaxis with a new NA needs further evaluation, while telbivudine should be considered in cases of renal dysfunction[89]. In HBV patients with CKD, new NAs are the best options to minimize the consequences of HBV infection, providing that their dosage is adjusted according to creatinine clearance and taking into account the potential nephrotoxicity and resistance profile. Thus, ETV and telbivudine, an agent with promising data showing improvement in creatinine clearance, seem to be the preferred choices in CHB patients with CKD, while TDF is considered the best option in patients with prior resistance to any nucleoside analog. Physicians should be aware that all HBsAg positive patients should be treated with NAs before RT to maintain undetectable HBV DNA and prevent hepatic decompensation after RT. In pregnant women with CHB, close monitoring is needed and in those with high HBV DNA (> 10^6 IU/mL); treatment with lamivudine, telbivudine or TDF in the last trimester of pregnancy is the preferred option to reduce the risk of HBV transmission. If an infected child ultimately develops CHB, antiviral treatment should not be started urgently, since most of them are in the immune-tolerant phase of the disease. All HBsAg-positive candidates for immunosuppressive therapy should receive NA(s) pre-emptively, regardless of baseline HBV DNA, up to 12 mo after cessation of immunosuppression. Finally, HBsAg negative/anti-HBc positive patients with hematological diseases and/or those who are going to receive rituximab-containing regimens, regardless of their anti-HBs and serum HBV DNA status, should be on anti-viral prophylaxis (Table 3).

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ORIGINAL ARTICLE

Basic Study

Immunohistochemical expression of SP-NK-1R-EGFR pathway and VDR in colonic inflammation and neoplasia

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Abstract

AIM: To determine the expression of neurokinin-1 receptor (NK-1R), phosphorylated epidermal growth factor receptor (pEGFR), cyclooxygenase-2 (Cox-2), and vitamin D receptor (VDR) in normal, inflammatory bowel disease (IBD), and colorectal neoplasia tissues from Puerto Ricans.

METHODS: Tissues from patients with IBD, colitis-associated colorectal cancer (CAC), sporadic dysplasia, and sporadic colorectal cancer (CRC), as well as normal controls, were identified at several centers in Puerto Rico. Archival formalin-fixed, paraffin-embedded tissues were de-identified and processed by immunohistochemistry for NK-1R, pEGFR, Cox-2, and VDR. Pictures of representative areas of each tissues diagnosis were taken and scored by three observers using a 4-point scale that assessed intensity of staining. Tissues with CAC were further analyzed by photographing representative areas of IBD and the different grades of dysplasia, in addition to the areas of cancer, within each tissue. Differences in the average age between the five patient groups were assessed with one-way analysis of variance and Tukey-Kramer multiple comparisons test. The mean scores for normal tissues and tissues with IBD, dysplasia, CRC, and CAC were calculated

and statistically compared using one-way analysis of variance and Dunnett's multiple comparisons test. Correlations between protein expression patterns were analyzed with the Pearson's product-moment correlation coefficient. Data are presented as mean ± SE.

RESULTS: On average, patients with IBD were younger (34.60 ± 5.81) than normal (63.20 ± 6.13, $P < 0.01$), sporadic dysplasia (68.80 ± 4.42, $P < 0.01$), sporadic cancer (74.80 ± 4.91, $P < 0.001$), and CAC (57.50 ± 5.11, $P < 0.05$) patients. NK-1R in cancer tissue (sporadic CRC, 1.73 ± 0.34; CAC, 1.57 ± 0.53) and sporadic dysplasia (2.00 ± 0.45) were higher than in normal tissues (0.73 ± 0.19). pEGFR was significantly increased in sporadic CRC (1.53 ± 0.43) and CAC (2.25 ± 0.47) when compared to normal tissue (0.07 ± 0.25, $P < 0.05$, $P < 0.001$, respectively). Cox-2 was significantly increased in sporadic colorectal cancer (2.20 ± 0.23 *vs* 0.80 ± 0.37 for normal tissues, $P < 0.05$). In comparison to normal (2.80 ± 0.13) and CAC (2.50 ± 0.33) tissues, VDR was significantly decreased in sporadic dysplasia (0.00 ± 0.00, $P < 0.001$ *vs* normal, $P < 0.001$ *vs* CAC) and sporadic CRC (0.47 ± 0.23, $P < 0.001$ *vs* normal, $P < 0.001$ *vs* CAC). VDR levels negatively correlated with NK-1R ($r = -0.48$) and pEGFR ($r = -0.56$) in normal, IBD, sporadic dysplasia and sporadic CRC tissue, but not in CAC.

CONCLUSION: Immunohistochemical NK-1R and pEGFR positivity with VDR negativity can be used to identify areas of sporadic colorectal neoplasia. VDR immunoreactivity can distinguish CAC from sporadic cancer.

Key words: Colitis; Colon cancer; Dysplasia; Neurokinin; Vitamin D

Core tip: This study compares for the first time the expression of neurokinin-1 receptor, phosphorylated epidermal growth factor receptor, cyclooxygenase-2, and vitamin D receptor in samples from patients with sporadic colorectal neoplasia, colitis, and colitis-associated colorectal neoplasia and obtained from a Puerto Rican population. We believe that this knowledge could be of great use for the further identification of reliable markers of cancer risk and of potential therapeutic targets in a population where colorectal cancer is the deadliest cancer, and that our findings could be used in the day-to-day examination of colonic biopsies for establishing a diagnosis and distinguishing dysplasia from reactive inflammatory changes.

Isidro RA, Cruz ML, Isidro AA, Baez A, Arroyo A, González-Marqués WA, González-Keelan C, Torres EA, Appleyard CB. Immunohistochemical expression of SP-NK-1R-EGFR pathway and VDR in colonic inflammation and neoplasia. *World J Gastroenterol* 2015; 21(6): 1749-1758 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i6/1749.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i6.1749

INTRODUCTION

Not only is colorectal cancer (CRC) the second most commonly occurring cancer among Puerto Rican men and women combined but it is also the deadliest cancer in the Puerto Rican population[1]. This situation contrasts with that of both the general United States population - where CRC is less frequent than prostate, breast, and lung cancer and less deadly than lung cancer - and in United States Hispanics - where CRC incidence is surpassed by that of prostate and breast cancer and more people die from lung cancer than from CRC[2,3]. Although the exact cause remains unknown, this disparity is likely due to a complex interplay between several factors, such as differences in genetic admixture and environment. Facilitating an accurate diagnosis of CRC and its precursor lesions in Puerto Ricans could help mitigate the disparate incidence and mortality of CRC.

CRC encompasses several different modalities of cancer, ranging from familial syndromes to colitis-associated malignancy to sporadic cancer. The latter two serve as an example of how the same pathology can arise through different pathways: sporadic CRC arises from adenomatous polyps that progress to carcinoma, also known as the adenoma-carcinoma sequence. In contrast, colitis-associated colorectal cancer (CAC) begins with the chronic inflammation characteristic of inflammatory bowel diseases (IBD), such as ulcerative colitis and Crohn's disease, and progresses to dysplasia and subsequently carcinoma, also known as the colitis-dysplasia-carcinoma sequence (Figure 1A)[4]. Dysplasia is a definite neoplastic change of the intestinal epithelium that does not penetrate the basement membrane[5-7]. The architectural changes that define colitis-associated dysplasia are analogous to those seen in adenomatous polyps[5]. Although dysplasia is currently the best marker of CRC risk in IBD, the wide range of variability between observers has led to a rising need for more reliable criteria and markers of cancer risk[8]. Interestingly, the prevalence of IBD in Puerto Rico has recently been estimated to be one of the highest among Hispanic groups[9], and Hispanics display phenotypic differences in the manifestations of IBD when compared to non-hispanic whites[10]. These two points emphasize the existence of differences not only between Hispanics and non-Hispanics but also between different Hispanic populations, thus indicating a need for studies that investigate disease processes in specific populations.

Much attention has been paid to signaling pathways and their components because of their involvement in carcinogenesis and their potential as therapeutic targets[11]. The substance P-neurokinin-1

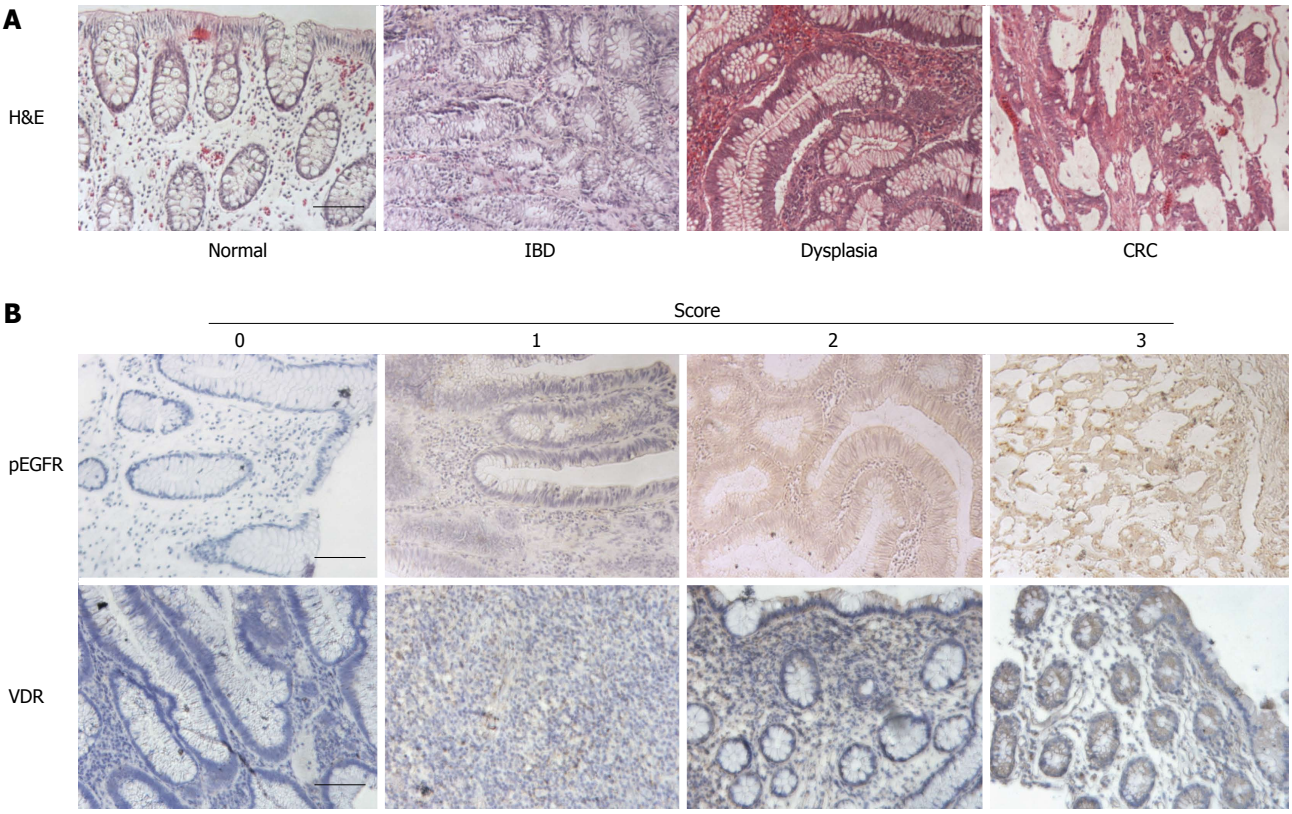

Figure 1 Histologic images depicting stages in the colitis-dysplasia-cancer progression of colitis-associated cancer. A: Colitis-associated colorectal cancer (CAC) is thought to arise through the chronic colitis-dysplasia-carcinoma sequence; B: Examples of immunohistochemistry scoring for phosphorylated epidermal growth factor receptor (pEGFR; top row) and vitamin D receptor (VDR; bottom row). A score of 0 indicates minimal or absent staining, whereas a score of 3 indicates strongest staining for a particular antibody. For pEGFR, the pictures scored as 0, 1, 2, and 3 correspond to tissues with a diagnosis of normal, inflammatory bowel disease (IBD), sporadic dysplasia, and sporadic colorectal cancer (CRC), respectively. For VDR, the pictures scored as 0, 1, 2, and 3 correspond to tissues with a diagnosis of sporadic dysplasia, sporadic colorectal cancer, IBD, and normal, respectively. Scale bars = 100 μm.

receptor-epidermal growth factor receptor (SP-NK-1R-EGFR) pathway is of particular interest, since previous studies from our laboratory have shown that signaling components involved in these pathways are up-regulated in rat models of chronic inflammation and colitis-associated neoplasia[12-15]. SP and its endogenous receptor, NK-1R, are known to play a pivotal role in the pathophysiology of intestinal inflammation and are also involved in many processes related to oncogenesis[16,17]. Binding of SP and NK-1R causes transactivation of the EGFR, with subsequent activation of other pathways, such as Raf/MEK/ERK and PI3K/PDK/Akt, which can lead to increased inflammation and proliferation, along with decreased apoptosis and differentiation[18,19]. A downstream product of several of these pathways is cyclooxygenase-2 (Cox-2), an isozyme involved in the synthesis of prostaglandins. The expression of Cox-2 is normally minimal under basal conditions but is increased when it is stimulated by inflammatory cytokines, growth factors, and endotoxins[20].

There has been increasing awareness regarding the possible importance of vitamin D levels in various cancers, including CRC[21]. Interestingly, binding of calcitriol (1,2-dihydroxycholecalciferol, the active form of vitamin D) to the vitamin D receptor (VDR) has been shown to block the EGFR and to inhibit several of its pathways (particularly, Raf/MEK/ERK), resulting in an increase in apoptosis and a decrease in proliferation and angiogenesis[22].

Previous studies have assessed the expression of several of the aforementioned signaling components in chronic colitis and in CRC. Levels of NK-1R, EGFR, and Cox-2 have been shown to be elevated both in patients with colitis and in patients with CRC[17,23-27]. VDR levels have been shown to be decreased in patients with colitis but either minimally expressed or over-expressed in CRC[21,28]. Importantly, little is known regarding the expression of these proteins during dysplasia. Furthermore, no study to date has investigated the expression pattern of these proteins in either Puerto Ricans or United States Hispanics. A unique expression pattern for these proteins in dysplasia could further elucidate their potentially critical roles in the development of cancer. Therefore, the aim of this study was to determine and compare the expression of NK-1R, pEGFR, Cox-2, and VDR in patients with sporadic and colitis-associated colorectal neoplasia and how it compares to that found in normal tissues and in patients with IBD, in samples obtained from a Puerto Rican population. This knowledge could be of great use for the further identification of reliable

Table 1 Patient sex and age distribution

Diagnosis	Sex	Mean age (range, yr)
Normal	3 M + 2 F	63.2 (42-75)[b]
IBD	4 M + 1 F	34.6 (23-56)
Dysplasia	3 M + 2 F	68.8 (52-76)[b]
Cancer	3 M + 2 F	73.6 (60-83)[b]
CAC	3 M + 3 F	57.5 (40-77)[a]

[a]$P < 0.05$, [b]$P < 0.01$, IBD patients *vs* normal, sporadic dysplasia, sporadic cancer, and CAC patients. CAC: Colitis-associated colorectal cancer; IBD: Inflammatory bowel disease; M: Male; F: Female (n = 5-6 patients per group).

Table 2 Colitis-associated colorectal cancer patient information

Sample ID	IBD diagnosis	Duration of disease (yr)	Sex	Age (yr)	Graph symbol
1	UC	9	M	61	●
2	UC	7	M	61	■
3	CD	20	F	49	▲
4	UC	27	F	40	▼
5	UC	35	M	57	◆
6	UC	57	F	77	★

Graph symbol indicates the unique symbol used to identify each patient in Figures 2B-5B. Duration of disease indicates duration at the time of biopsy. IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn's disease.

markers of cancer risk and of potential therapeutic targets in a population where CRC is the deadliest cancer.

MATERIALS AND METHODS

Use of human subjects and Institutional Review Boards approval

This study was carried out in compliance with all NIH regulations concerning the Protection of Human Subjects and human tissue specimens were approved for use by Institutional Review Boards (IRB) as follows. The Ponce School of Medicine and Health Sciences Institutional Review Board approved this study under IRB protocol 080506-CA and the University of Puerto Rico Medical Sciences Campus Institutional Review Board approved this study under IRB protocol MSC IRB 1250112. Both institutional review boards waived the need for informed consent given that the samples were existing archived pathological specimens which were de-identified, and thus did not contain any identifiable information that could be linked to the subjects.

Patients and tissue samples

Archived pathology laboratory samples, embedded in paraffin and devoid of personal information, were obtained from pathology laboratories of local hospitals. Samples (paraffin blocks) of colonic tissue from 26 different patients were randomly selected by laboratory staff for each of the following diagnoses: normal (n = 5), IBD (n = 5), dysplasia (n = 5), cancer (n = 5) and CAC (n = 6; Table 1). Patients with a histopathologic diagnosis of CAC in biopsy or surgical specimens were identified in the UPR Center for Inflammatory Bowel Diseases database (Table 2). Corresponding archived paraffin-embedded tissue blocks were retrieved from UPR School of Medicine Pathology Laboratory and de-identified by two pathologists (WGM and CGK), with the identification list saved for re-identification. All blocks were transferred to the Gastrointestinal Research Laboratory at Ponce School of Medicine and Health Sciences. Tissue sections from each of the selected samples were stained with hematoxylin and eosin in order for an independent pathologist (AAI) to confirm the diagnosis.

Immunohistochemistry

Tissue sections were deparaffinized in two 15-min Hemo-De xylene-substitute baths. Graded ethanol dilutions and distilled water were used for tissue rehydration. Hydrogen peroxide (3%, aqueous) was used to block endogenous peroxidase activity. After washing with phosphate-buffered saline, antigen retrieval was achieved by placing sections in citrate-EDTA buffer (10 mmol/L; 2 mmol/L EDTA, 0.05% Tween 20, pH 6.2) at 95℃-99℃ for 40 min and at room temperature for 20 min. Sections were then washed with distilled water for 2 min (twice), phosphate-buffered saline for 5 min, and normal serum (Biogenex, San Ramon, CA) for 15 min before overnight incubation with the primary antibody. Antibodies used included NK-1R (sc-15323; Santa Cruz Biotechnology, dilution 1:100), pEGFR (#2236; Cell Signaling Technology, dilution 1:400), Cox-2 (#160106; Cayman Chemical Co., dilution 1:200), and VDR (Ab3508; Abcam Inc, dilution 1:2000). Secondary antibody was added to the sections for 20 min using the Super Sensitive Link-Label IHC Detection System (Biogenex, San Ramon, CA); sections were incubated with the biotinylated secondary antibody for 20 min and then with a streptavidin-peroxidase conjugate for 20 min. 3,3-Diaminobenzidine chromogen solution (Biogenex, San Ramon, CA) was used to achieve color development. Tissue sections were counterstained with hematoxylin for 20 s, dehydrated with graded ethanol dilutions, cleared with xylene, and mounted with a xylene-based mounting medium. Pictures were taken of representative areas containing the diagnosis of interest for each sample and blindly scored by three observers. A four-point scale ranging from 0 to 3 was used to assign scores according to staining intensity. In this scale, a score of 0 corresponds to the lightest, or absence of, staining, whereas 3 corresponds to the strongest staining for a particular antibody (Figure 1B). Additionally, areas diagnostic of IBD, dysplasia (mild, moderate, and severe), and cancer in tissue from CAC patients were photographed and scored.

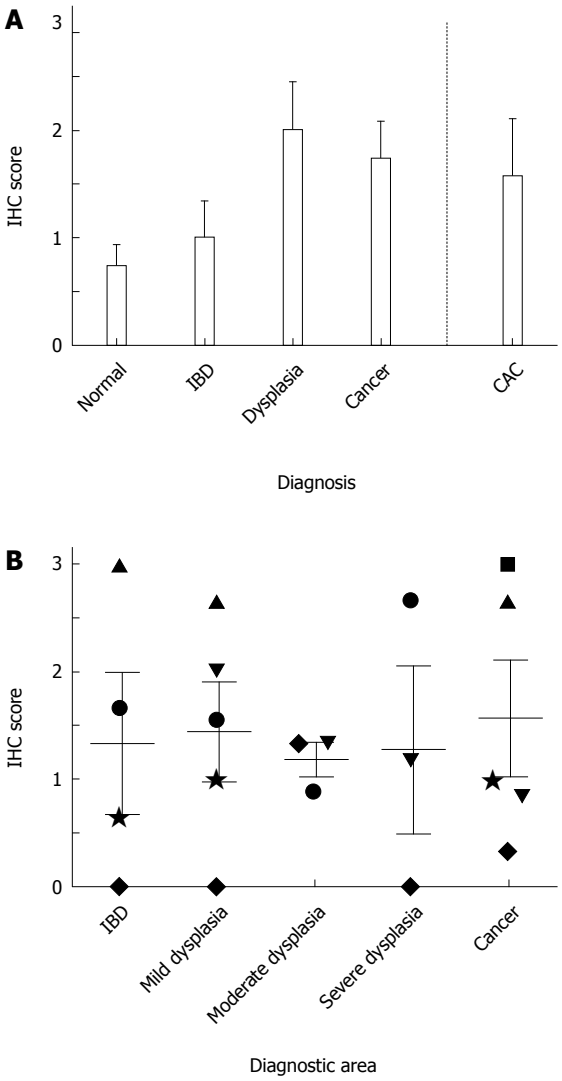

Figure 2 Expression of neurokinin-1 receptor in colonic tissue. A: Neurokinin-1 receptor (NK-1R) expression in tissue from normal, inflammatory bowel disease (IBD), sporadic dysplasia, sporadic colorectal cancer, and colitis-associated colorectal cancer (CAC) patients (n = 5 patients per group); B: Expression of NK-1R in different diagnostic areas found within tissue from CAC patients. Each patient is identified by a unique symbol (Table 2). IHC: Immunohistochemistry.

Statistical analysis

Graphpad Prism V6.0a (Graphpad Software, San Diego, CA) was used to perform all statistical analyses. One-way analysis of variance and post hoc multiple comparisons tests were used to determine the statistical significance of the measured variables. A Tukey-Kramer multiple comparisons test was used to analyze differences between age distributions in the five patient groups. Dunnett's multiple comparisons tests were used to analyze differences in protein expression. Pearson's product-moment correlation coefficient was used to analyze the associations between expression of the four proteins measured. A difference was considered significant if $P < 0.05$. Data presented as mean ± SE.

RESULTS

There were no significant differences in gender between groups. As might be expected, IBD patients were significantly younger than normal, sporadic dysplasia, sporadic cancer, and CAC patients ($P < 0.01$, $P < 0.01$, $P < 0.001$, and $P < 0.05$ respectively; Table 1). There were no significant differences in age between any of the other groups.

NK-1R levels are increased in colonic neoplasia

All diagnoses showed higher NK-1R expression when compared to normal tissues, although this failed to reach statistical significance (Figure 2A). NK-1R levels doubled in sporadic cancer and CAC when compared to normal. In normal tissues, staining was found in the glands and surface epithelium. In IBD, staining was found predominantly in the columnar cells of the glands, with some additional staining in the surface epithelium (in 40% of the samples). Sporadic dysplasia stained predominantly in the cytoplasm of columnar cells of the glands, whereas in sporadic cancer the staining was mostly found in the cytoplasm of poorly differentiated cancer cells. In CAC, staining was mainly observed in the cytoplasm of transformed glandular epithelial cells, and did not vary significantly between different diagnostic areas (Figure 2B).

pEGFR levels are significantly increased in sporadic and colitis-associated colorectal cancer

For pEGFR, a 15-fold increase and a 19-fold increase in expression were observed in IBD (1.00 ± 0.10) and sporadic dysplasia (1.27 ± 0.34), respectively, when compared to normal patients (0.07 ± 0.07; Figure 3A). Patients with sporadic cancer or CAC had significantly higher expression levels than those found in normal tissues ($P < 0.05$ and $P < 0.001$, respectively). In normal tissues, minimal to no staining was found, with only one sample showing nonspecific staining in the surface epithelium. Staining in IBD, sporadic dysplasia, sporadic cancer, and CAC tissues was observed in the columnar cells of the glands, mainly in the cytoplasm. Areas of cancer within CAC tissue had higher levels of pEGFR than other diagnostic areas present, but these differences were not statistically significant (Figure 3B). Figure 1B (top row) shows an example of pEGFR staining.

Cox-2 levels are significantly increased in sporadic colorectal cancer

Cox-2 expression had an increasing trend similar to that of pEGFR, with higher expression occurring with worsening diagnosis. In patients with sporadic cancer, levels more than doubled (2.20 ± 0.23) and were significant when compared with normal tissues ($P < 0.05$; Figure 4A). Cox-2 levels in CAC tissue were

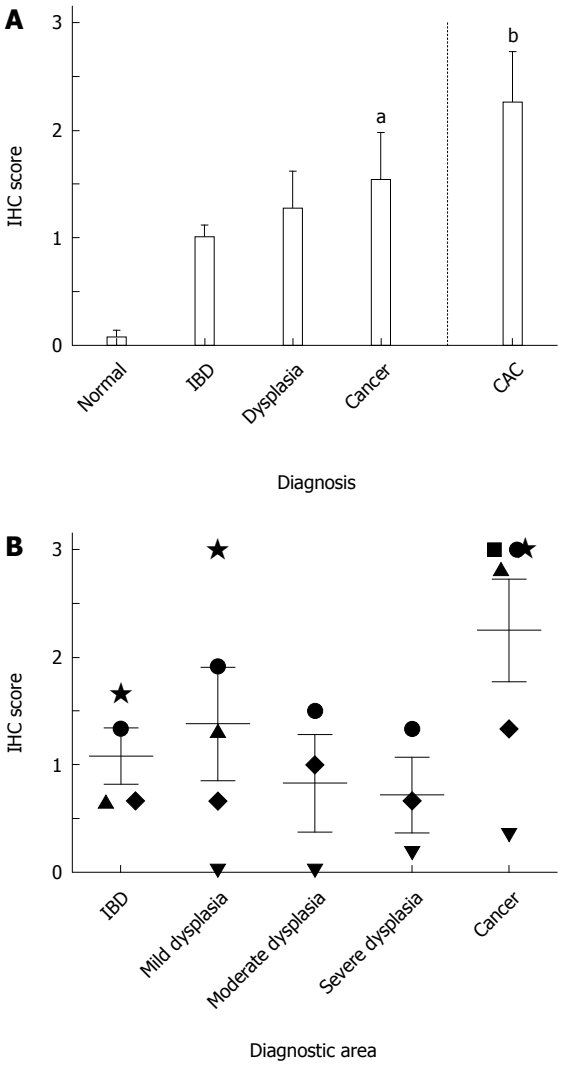

Figure 3 Expression of phosphorylated epidermal growth factor receptor in colonic tissue. A: Phosphorylated epidermal growth factor receptor (pEGFR) expression in tissue from normal, inflammatory bowel disease (IBD), sporadic dysplasia, sporadic colorectal cancer, and colitis-associated colorectal cancer (CAC) patients (n = 5 patients per group except CAC, where n = 6). [a]$P < 0.05$ and [b]$P < 0.01$ *vs* normal; B: Expression of pEGFR in different diagnostic areas found within tissue from CAC patients. Each patient is identified by a unique symbol (see Table 2). IHC: Immunohistochemistry.

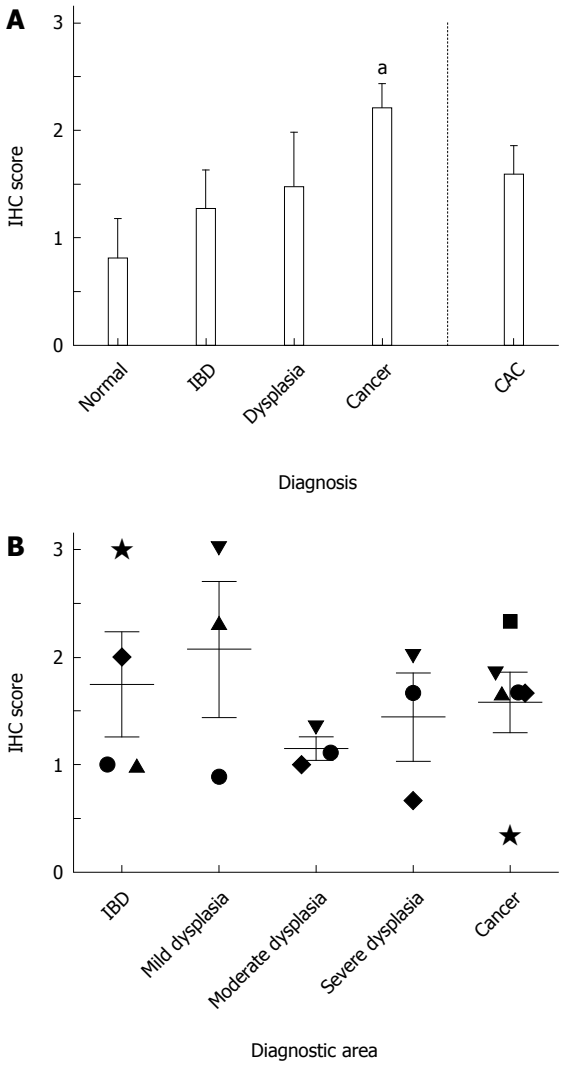

Figure 4 Expression of cyclooxygenase-2 in colonic tissue. A: Cyclooxygenase-2 (Cox-2) expression in tissue from normal, inflammatory bowel disease (IBD), sporadic dysplasia, sporadic colorectal cancer, and colitis-associated colorectal cancer (CAC) patients (n = 5 patients per group except CAC, where n = 6). [a]$P < 0.05$ *vs* normal; B: Expression of Cox-2 in different diagnostic areas found within tissue from CAC patients. Each patient is identified by a unique symbol (Table 2). IHC: Immunohistochemistry.

not as elevated as in tissues with sporadic cancer. No significant differences were found between the other groups. In normal tissues, staining was observed in the glands and surface epithelium, when present. Tissues from patients with IBD, sporadic dysplasia, sporadic cancer, and CAC showed staining in the columnar cells of the glands, mainly in the cytoplasm. In CAC tissues, Cox-2 expression did not vary significantly between different diagnostic areas (Figure 4B) and negatively correlated with increased disease duration (r = -0.87, $P < 0.05$).

VDR levels are significantly decreased in sporadic neoplasia of colonic epithelium

The highest levels of VDR expression were found in normal tissues. In patients who had been diagnosed with sporadic dysplasia, no expression was found ($P < 0.001$ *vs* normal), with minimal VDR expression occurring in sporadic CRC ($P < 0.001$ *vs* normal; Figure 5A). VDR levels in CAC tissue were significantly higher than those of sporadic dysplasia and cancer ($P < 0.001$) and more similar to those found in IBD and normal tissue. Normal tissues stained in the cytoplasm of the columnar cells of the glands. In IBD tissues, staining was found in the cytoplasm of the columnar cells of the glands and the acute inflammatory cell infiltrate. In sporadic cancer, 4 out of 5 tissues had no staining in the glands; 3 of these had light staining limited to inflammatory infiltrate within the areas of necrosis. Only 1 of the samples had slight staining in the columnar cells of the glands. In CAC, staining was mostly in the cytoplasm of glandular epithelial cells and inflammatory lamina

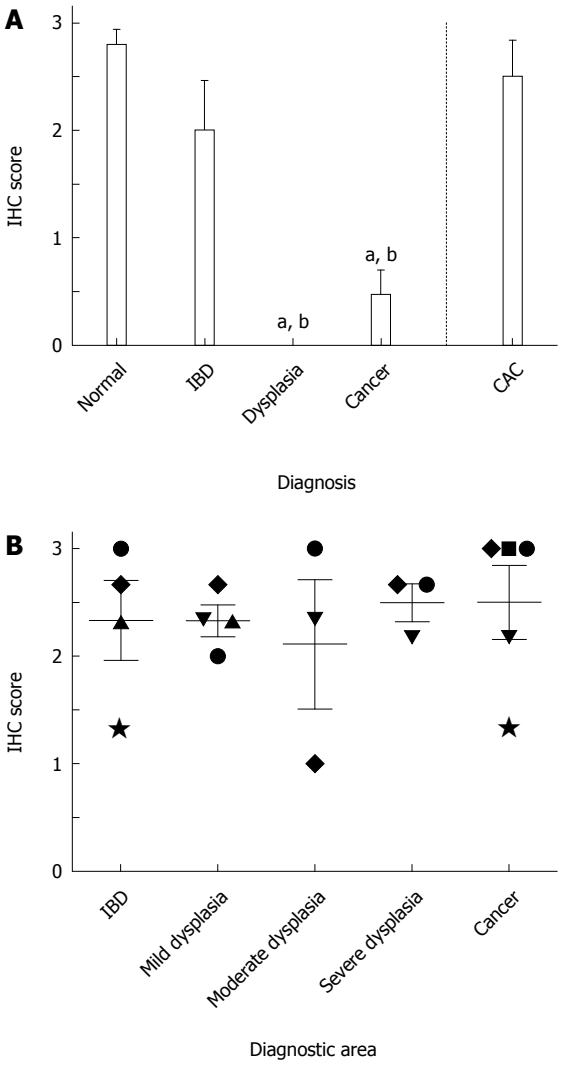

Figure 5 Expression of vitamin D receptor in colonic tissue. A: Vitamin D receptor (VDR) expression in tissue from normal, inflammatory bowel disease (IBD), sporadic dysplasia, sporadic colorectal cancer, and colitis-associated colorectal cancer (CAC) patients (n = 5 patients per group except CAC, where n = 6). $^{a}P < 0.001$ *vs* normal and $^{b}P < 0.001$ *vs* CAC; B: Expression of VDR in different diagnostic areas found within tissue from CAC patients. Each patient is identified by a unique symbol (see Table 2). IHC: Immunohistochemistry.

propria cells and was consistent across the different diagnostic areas (Figure 5B). Figure 1B (bottom row) shows an example of VDR staining.

VDR levels negatively correlate with NK-1R and pEGFR levels in colonic epithelium

Statistical tests showed that VDR expression had a significant negative correlation with NK-1R (r = -0.48) and pEGFR (r = -0.56) expression ($P < 0.05$) when CAC tissue was excluded. These associations were lost when analysis included results from CAC tissue.

DISCUSSION

Our laboratory has previously studied the role of NK-1R, EGFR, Cox-2, and VDR in a rat model of colitis-associated dysplasia and cancer[12]. We demonstrated that antagonizing either the NK-1R or the EGFR delayed progression to dysplasia, decreased Cox-2 levels, and reduced inflammation[13,14]. Also, we demonstrated that delaying the transition to dysplasia by administering probiotics was associated with an increase in VDR expression[29]. In the present study, we report the comparative expression patterns of NK-1R, EGFR, Cox-2, and VDR in colonic tissue from Puerto Rican patients with IBD, sporadic colorectal neoplasia, and colitis-associated colorectal neoplasia.

Prior reports have demonstrated that there is an alteration in the expression of NK-1R, pEGFR, Cox-2, and VDR both in chronic colitis and in neoplasm of the colon. However, the expression of these proteins in Hispanic patients has never been studied. Furthermore, NK-1R has been found to be increased in tissue from patients with IBD and colon cancer[30,31]. Here, we showed that NK-1R levels were increased in colonic inflammation and neoplasia samples from Puerto Rican patients, although these results were not significantly different, most likely due to the small sample size. Finding tissue from Puerto Rican patients with colitis-associated dysplasia or cancer was extremely difficult. After 2 years of actively seeking said tissue, we were able to find only those tissues that were used in this study. Curiously, NK-1R levels in sporadic dysplasia did reach statistical significance, but only if the CAC group was excluded. Nevertheless, our results suggest that NK-1R is a relevant player in colonic inflammation and neoplasia in Puerto Rican patients. Gillespie *et al*[32] demonstrated that a truncated form of NK-1R, rather than its full-length counterpart, is increased in colectomy tissue from patients with CAC who were treated at the Boston University Medical Center. Our present study, however, seems to indicate that levels of the full-length NK-1R were increased in both sporadic and colitis-associated colorectal neoplasia tissue samples from Puerto Rican patients.

The role of EGFR in inflammatory and malignant pathologies of the colon has long been known: Sottili *et al*[24] demonstrated that EGFR is upregulated in experimental colitis, and Malecka-Panas *et al*[25] demonstrated that EGFR is increased in the colonic mucosa of patients with ulcerative colitis, adenomatous polyps, and CRC, The relevance of EGFR in CRC is such that anti-EGFR antibodies are currently being used as adjuvant therapy in metastatic CRC. Our current findings in samples from Puerto Rican patients are consistent with the aforementioned studies: pEGFR is increased in colonic inflammation and neoplasia, with significantly elevated levels found in tissue from both sporadic CRC and CAC. It is interesting to note that the increased pEGFR expression does not exactly match the increase in NK-1R levels. This suggests that a factor other than NK-1R may account for the marked increase seen in CAC. One possibility is that, in Puerto Rican patients, EGFR might become an oncogene in the colitis-dysplasia-carcinoma sequence

of CAC through a mechanism different from that which takes place in the adenoma-carcinoma sequence of sporadic CRC. Our present findings also suggest that therapies directed against EGFR might be of benefit to Puerto Rican CAC patients, thus warranting further investigation. Our findings also underscore the necessity to include different ethnic groups in clinical trials, since these may respond differently to the same treatment.

In ulcerative colitis, Cox-2 expression is up-regulated in the inflamed mucosa, and there is evidence showing an overexpression of Cox-2 in cancer tissues, suggesting an important role in tumorigenesis[26,27,33,34]. Several epidemiological studies have found that these levels are reduced in individuals with CRC who are taking non-steroidal anti-inflammatory drugs[35]. Our findings show that, although expression is increased in tissue from IBD and CAC patients, Cox-2 levels are highest in sporadic CRC. Intriguingly, Cox-2 levels in CAC patients negatively correlated with disease duration, leading us to speculate that medications used to ameliorate the inflammation of IBD could alter the expression of Cox-2. Unfortunately, we were unable to confirm this suspicion due to the limited patient information available to us.

VDR is expressed in early-stage neoplasias but is repressed in high-grade and metastatic cancers[28,36]. Wada *et al*[21] (2009) demonstrated that VDR expression decreases in ulcerative colitis, dysplasia, and cancer. We have also shown that, in Puerto Rican patients, there is a significant decrease in VDR expression in sporadic dysplasia and in CRC, but not in CAC, when compared to normal tissue. In sporadic CRC, it is highly likely that without VDR, calcitriol cannot regulate the EGFR-related pathways, resulting in uncontrolled proliferation in the absence of differentiation and apoptosis[22]. Although the exact cause of the reduced VDR expression is yet to be elucidated, one possibility is that cellular signaling events may culminate in the nuclear translocation of certain factors that inhibit the transcription of the VDR gene. A possible candidate is SNAIL, a transcription factor that represses VDR expression and whose upregulation in colonic tumors has been associated with VDR downregulation[37]. Intriguingly VDR levels in CAC were not significantly lower than those seen in normal or IBD tissues. Although it has been reported that VDR can negatively regulate EGFR-related pathways[22], this does not seem to be the case in CAC, since both VDR and pEGFR are overexpressed. Perhaps another factor is affecting VDR expression in CAC. Although vitamin D deficiency is common among patients with IBD[38], the effect of this deficiency on colonic VDR expression is not clear. A possible explanation may lie in the different molecular mechanisms responsible for the development of CAC and sporadic CRC. Cancer-associated mutant p53 can affect VDR-mediated transcriptional activation and repression[39]. Both CAC and sporadic CRC tend to have p53 mutations; however, these mutations tend to occur at later stages in sporadic CRC and at very early stages in CAC[4]. Therefore, the long-term effects of mutant p53 could be responsible for the increased VDR levels seen in Puerto Rican patients with CAC.

To our knowledge, this study is the first to address NK-1R, pEGFR, Cox-2, and VDR expression in Puerto Ricans or any other Hispanic group. It is also one of the few studies to examine and compare changes in expression for these proteins in sporadic and colitis-associated neoplasia. Doing so allowed us to determine the expression pattern for these proteins in IBD, sporadic dysplasia, sporadic CRC, and CAC. Of particular interest is the differential expression of pEGFR and VDR in sporadic CRC. Negative or weak immunohistochemical staining for VDR and strong immunohistochemical staining for pEGFR in colonic gland cytoplasm in conjunction with architectural changes can be very useful in distinguishing areas of sporadic colonic dysplasia from cancer and from non-neoplastic changes. These stains can further help the pathologist pinpoint the regions in which the presence of atypical mitosis and/or invasion will render a diagnosis of malignancy, and absence of these findings will establish the diagnosis of dysplasia. This should greatly aid in reducing inter-observer variability in establishing these crucial diagnoses, and, by doing so, may help mitigate the current disparity in CRC incidence and mortality between Puerto Ricans and the general United States population. Unfortunately, these changes are not as useful in colitis-associated neoplasia. Whether these findings are limited to Puerto Rican patients or generalizable to other Hispanic groups or the general population remains to be determined. Finally, although inflammation is involved in sporadic neoplasia[40] as well as in colitis-associated colorectal neoplasia, our findings further emphasize the different molecular events underlying the pathogenesis of sporadic CRC and CAC.

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COMMENTS

Background

Colorectal cancer (CRC) is the deadliest cancer in the Puerto Rican population and the second deadliest cancer in both the general United States population and in United States Hispanics. Signaling components of the substance

P (SP)-neurokinin-1 receptor (NK-1R)-epidermal growth factor receptor (EGFR) pathway are involved in development of intestinal inflammation and in processes related to oncogenesis. Calcitriol and the vitamin D receptor (VDR) can regulate EGFR and its pathways and may antagonize the neoplastic process.

Research frontiers

Expression of these signaling components has not been examined in Puerto Ricans or United States Hispanics, populations where colorectal cancer is one of the deadliest cancers, in colon tissues with cancer and/or with the different histopathological changes that lead up to cancer. This knowledge might be important for CRC prevention and treatment.

Innovations and breakthroughs

This is the first study to address the expression of NK-1R, pEGFR, Cox-2, and VDR in colonic tissue from Puerto Rican patients. In these patients, pEGFR was significantly increased in sporadic and colitis-associated colorectal cancer (CAC), Cox-2 was significantly increased in sporadic CRC, and VDR was significantly decreased in sporadic dysplasia and sporadic CRC. Overall, VDR levels negatively correlated with NK-1R and pEGFR in tissues without CAC.

Applications

These findings suggest that, in these patients, NK-1R and pEGFR immunohistochemical positivity together with VDR immunohistochemical negativity could be used to identify areas of neoplastic change in sporadic colorectal neoplasia, with changes in VDR immunoreactivity distinguishing CAC from sporadic cancer.

Terminology

Sporadic CRC is the type of cancer of the colon and rectum that tends to develop in the general population after the age of 50. In contrast, CAC is a type of colorectal cancer that arises in patients with inflammatory bowel disease (IBD). The risk of developing CAC increases with the duration, extent, and severity of colonic inflammation. Dysplasia of the colon and rectum is defined as a neoplastic change of the epithelium that is not invasive, and therefore non-cancerous or benign. Nonetheless, dysplastic epithelium may eventually become cancerous, and therefore malignant and invasive. Neoplasia includes both benign changes, such as dysplasia, and malignant changes, such as CRC.

Peer-review

In this article, the authors compared for the expression of NK-1R, pEGFR, Cox-2, and VDR with sporadic colorectal neoplasia, colitis, and colitis-associated colorectal neoplasia from a Puerto Rican population. The findings suggest that NK-1R and pEGFR immunohistochemical positivity together with VDR immunohistochemical negativity could be used to identify areas of neoplastic change in sporadic colorectal neoplasia, with changes in VDR immunoreactivity distinguishing CAC from sporadic cancer. This is a well-written paper containing interesting results which merit publication.

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ORIGINAL ARTICLE

Basic Study

Beta-7 integrin controls enterocyte migration in the small intestine

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Abstract

AIM: To hypothesize that beta-7 integrin affects cellular migration of both, lymphocytes and enterocytes.

METHODS: The nucleoside analog BrdU was *ip* injected in beta-7-deficient mice (C57BL/6-Itgb^{tmlcgn}/J) of male gender and age-matched male C57BL/J J mice (wild type) 4, 20, or 40 h before analysis. The total small intestine was isolated, dissected, and used for morphometrical studies. BrdU-positive epithelial cells were numbered in at least 15 hemi-crypts per duodenum, jejunum, and ileum of each animal. The outer most BrdU-positive cell (cell^{max}) was determined per hemi-crypt, numerically documented, and statistically analysed.

RESULTS: Integrins containing the beta-7-chain were exclusively expressed on leukocytes. In the small intestinal mucosa of beta-7 integrin-deficient mice the number of intraepithelial lymphocytes was drastically decreased. Moreover, the Peyer's patches of beta-7 integrin-deficient mice appeared hypoplastic. In beta-7 integrin-deficient mice the location of cell^{max} was found in a higher position than it was the case for the controls. The difference was already detected at 4 h after BrdU application, but significantly increased with time (40 h after BrdU injection) in all small intestinal segments investigated, *i.e.*, duodenum, jejunum, and ileum. Migration of small intestinal enterocytes was different between the experimental groups measured by cell^{max} locations.

CONCLUSION: The E-cadherin beta-7 integrin pathway probably controls migration of enterocytes within the small intestinal surface lining epithelial layer.

Key words: Barrier function; Cell migration; Inflammatory bowel disease; Integrin; Intercellular junctions

Core tip: Integrins are involved in migration of epithelial

and non-epithelial cells. beta-7-chain integrins are exclusively expressed on leukocytes and important for homing of lymphocytes into the intestinal mucosa. In beta-7 integrin-deficient mice, the number of intraepithelial lymphocytes is drastically decreased and accompanied by a significant increase in enterocyte migration along the crypt-villus axis. This phenomenon is probably mediated by the E-cadherin beta-7 integrin pathway.

Kaemmerer E, Kuhn P, Schneider U, Clahsen T, Jeon MK, Klaus C, Andruszkow J, Härer M, Ernst S, Schippers A, Wagner N, Gassler N. Beta-7 integrin controls enterocyte migration in the small intestine. *World J Gastroenterol* 2015; 21(6): 1759-1764 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i6/1759.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i6.1759

INTRODUCTION

Intestinal surface lining epithelial cells include enterocytes, goblet cells, enterochromaffine cells, M-cells, and Paneth cells. All of these cell types are continuously replaced within a couple of days. This process is supported by cellular migration along the crypt-villus axis (CVA) or crypt-plateau axis[1-3]. Enterocyte migration essentially contributes to configuration and homeostasis of the intestinal mucosal barrier and plays a pivotal role in mucosal healing after inflammatory or non-inflammatory injuries[4]. It is suggested that in the intestine differentiation and behaviour of epithelial cells are modified by lymphoid cells[5,6].

A plethora of molecular mechanisms and pathways is involved in coordination of epithelial and non-epithelial cell movement[7,8]. Important steps in cellular migration are G-protein-mediated cellular polarisation with local increase in phosphatidylinositol-3,4,5-triphosphate, activation of Rho GTPases, and a synergism of actin polymerisation with the Wiskott-Aldrich syndrome protein/WASP-family verprolin-homologous protein and Arp2/3-complex forming lamellipodia, filopodia, podosomes or invadopodia[9-12].

Integrins are *trans*-membrane cell adhesion receptors connecting the extra- and intracellular environments by binding to ligands or structures outside the cell and cytoskeletal components or signalling molecules inside the cell[13]. They are heterodimers, consisting of non-covalently associated alpha and beta chains. The composition of diverse alpha and beta subunits determines the plasticity and diversity in ligand recognition and signalling of integrins[14]. The integrin binding specificity is determined by the extracellular domain recognizing diverse matrix ligands including fibronectin, collagen, and laminin. Modification of the integrin heterodimer repertoire can cause altered cellular migration, including more invasive phenotypes[8]. Size, morphology, and location of integrin-based adhesions strongly depend on the cell type and its environment. Several beta subunits are preferentially expressed by immune cells. Especially, beta-2 and beta-7 integrins are exclusively found on leukocytes[13].

Beta-7 integrin can form heterodimers with either the alpha4 or the alphaE subunit. Beta-7 integrins were characterized as important molecular structures in formation of the gut-associated lymphoid tissue (GALT)[15]. Alpha-4 beta-7 integrin binds to mucosal addressin cell adhesion molecule-1 (MAdCAM-1), which is constitutively expressed on intestinal endothelial cells and facilitates the retention of lymphocytes in the gut epithelium *via* E-cadherin[16]. Whereas beta-7 integrin-deficient mice display a significant reduction of intra-epithelial lymphocytes (IELs), lamina propria lymphocytes, hypoplasia of Peyer's patches, and a reduced number of IgA- and IgM-producing cells in the small intestine, alphaE integrin-deficient mice exhibit reduced numbers of IELs[17].

Here we concentrate on a detailed evaluation of the impact of beta-7 integrin on enterocyte migration in the small intestinal mucosa of mice.

MATERIALS AND METHODS

Beta-7 integrin-deficient mice

For the present study beta-7-deficient mice (C57BL/6-Itgb^{tmlcgn}/J[15]) of male gender and age-matched male C57BL/J J mice (wild type) were used. All animals were bred at the core facility of the University Hospital Aachen under specific pathogen-free conditions. In order to analyse intestinal epithelial cellular migration, all mice received a single ip injection of the nucleoside analog BrdU (30 μg/g mouse; Applichem, Cheshire, CT) 4, 20, or 40 h before sacrificing. All procedures were approved by the authority for environment conservation and consumer protection of the state North Rhine-Westfalia (LANUV, Germany).

Tissue preparation

After sacrificing, the total small intestine was isolated and dissected. Afterwards, the intestinal tissues (duodenum, jejunum, and ileum) were fixed for 24 h in neutral buffered formalin and automatically processed to paraffin. A binocular loupe was used for orthogonal orientation of tissues in paraffin. From each paraffin-embedded tissue block, sections of 3-5 μm were performed and stained with hematoxylin-eosin (HE).

Tissue morphometry

For morphometric procedures, tissue sections were inspected with a Nikon Eclipse 80i microscope (Nikon, Düsseldorf, Germany). Up to 50 hemi-crypts per animal were evaluated (about 15 hemi-crypts per duodenum, jejunum, and ileum). Criteria for small intestinal hemi-crypts were adapted from a previously published study[18]: (1) a single epithelial layer is visible from crypt basis to villus tip; (2) crypt basis

without any distension; (3) open crypt lumen; (4) between crypt and villus a small plateau is visible; (5) crypt height 1/4 to 1/3 of the total CVA; and (6) in each villus, the lamina propria mucosae is visible.

Immunohistochemistry

Serial sections of morphologically proved intestinal tissues were prepared and immunostained against BrdU[2]. After blocking, DNA-incorporated BrdU was detected with anti-BrdU antibodies (GE Healthcare, Freiburg, Germany). Antibody-binding was visualized with the MOM Kit and DAB as a chromogen (both from Vector, Burlingame, United States). Anti-CD3 immunostainings were performed using a rabbit polyclonal anti-CD3 antibody (Abcam, Cambridge, United Kingdom) in a dilution 1:100. For antibody detection the ImmPRESS system (Vector, Burlingame, CA) and DAB as chromogen were applied.

Measurement of epithelial cell migration

For each animal BrdU-positive, epithelial cells were numbered in 50 hemi-crypts per animal along the small intestine (about 15 hemi-crypts per duodenum, jejunum, and ileum) following previously published procedures[1,2]. BrdU-positive epithelial cells were characterized with "1", BrdU-negative epithelial cells with "0". In each hemi-crypt, the outer most BrdU-positive cell (cell^{max}) was determined and numerically documented. Histomorphological evaluations were performed by two morphologically experienced investigators (Kuhn P and Gassler N) using a Nikon Eclipse 80i microscope (Nikon).

Statistical analysis

For each time point of the study three animals per group (n_{ij} = 3) were used. Data of 50 hemi-crypts per small intestine (about 15 hemi-crypts per duodenum, jejunum, and ileum) and animal were summarized and the median was calculated. The same data were used for the analysis of variance reflecting mouse type and time course (4, 20 or 40 h). Data were processed using Excel-based algorithms and SAS-based procedures. Standard deviations of the means were indicated by error bars. *P*-values of less than 0.05 were considered significant.

RESULTS

Diminished intra-epithelial lymphocytes in beta-7 integrin-deficient mice

Using HE-stained tissue sections, small intestinal histomorphology of beta-7 integrin-deficient mice and wild types littermates was compared. In both groups, the basal tissue architecture was preserved throughout the small intestine (duodenum, jejunum, and ileum) including mucosa, submucosa, muscularis propria, and serosa. However, the number of IELs was significantly reduced in all small intestinal segments of beta-7 integrin-deficient mice when compared to controls (Figure 1). In addition, the Peyer's patches of beta-7 integrin-deficient mice appeared hypoplastic. These findings were in high accordance with the original description of an injured GALT in beta-7 integrin-deficient mice[15].

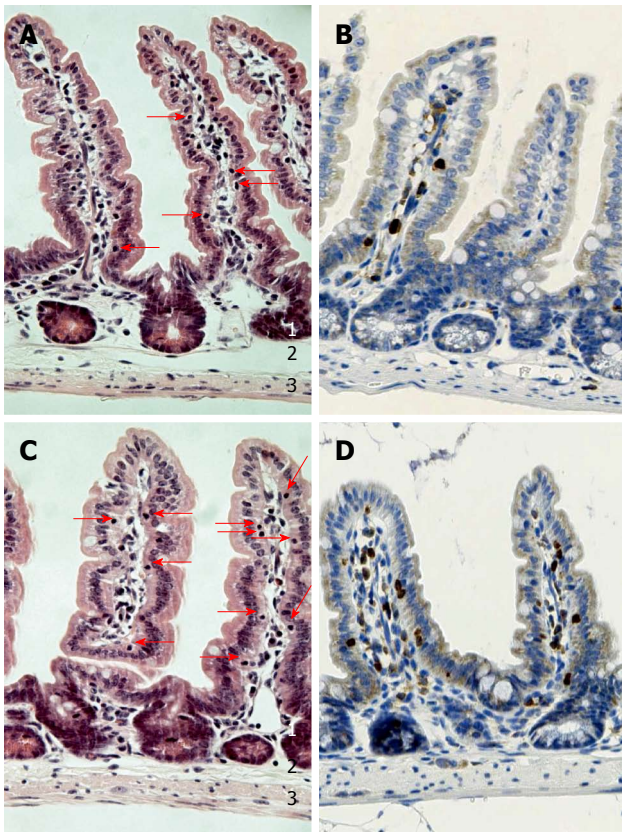

Figure 1 Number of intra-epithelial lymphocytes is diminished in beta-7 integrin-deficient mice. Histological sections of the jejunum from beta-7 integrin-deficient mice (A and B) as well as age-matched male C57BL/J J mice (C and D) are shown. In the HE stainings (A and C) characteristic intra-epithelial lymphocytes are highlighted by arrows. Anti-CD3 immunostainings are shown (B and D). Original magnification, × 400. 1: Tunica mucosa; 2: Tela submucosa; 3: Muscularis propria.

Increased enterocyte migration in beta-7 integrin-deficient mice

To evaluate migration of small intestinal enterocytes *in vivo* we used the BrdU-assay established by Potten *et al*[1,2]. Beta-7 integrin-deficient mice and controls were sacrificed 4, 20 or 40 h after BrdU application. Location of cell^{max} along the CVA was morphometrically evaluated in 50 hemi-crypts throughout the small intestine of each animal (about 15 hemi-crypts per duodenum, jejunum, and ileum) (Figure 2).

In the small intestinal mucosa of beta-7 integrin-deficient mice, the location of cell^{max} was found in a significantly higher position than it was the case for the controls (Figure 3). The difference was already detected at 4 h after BrdU application, but significantly increased with time (40 h after BrdU injection; *P*-value < 0.05). The differences observed were

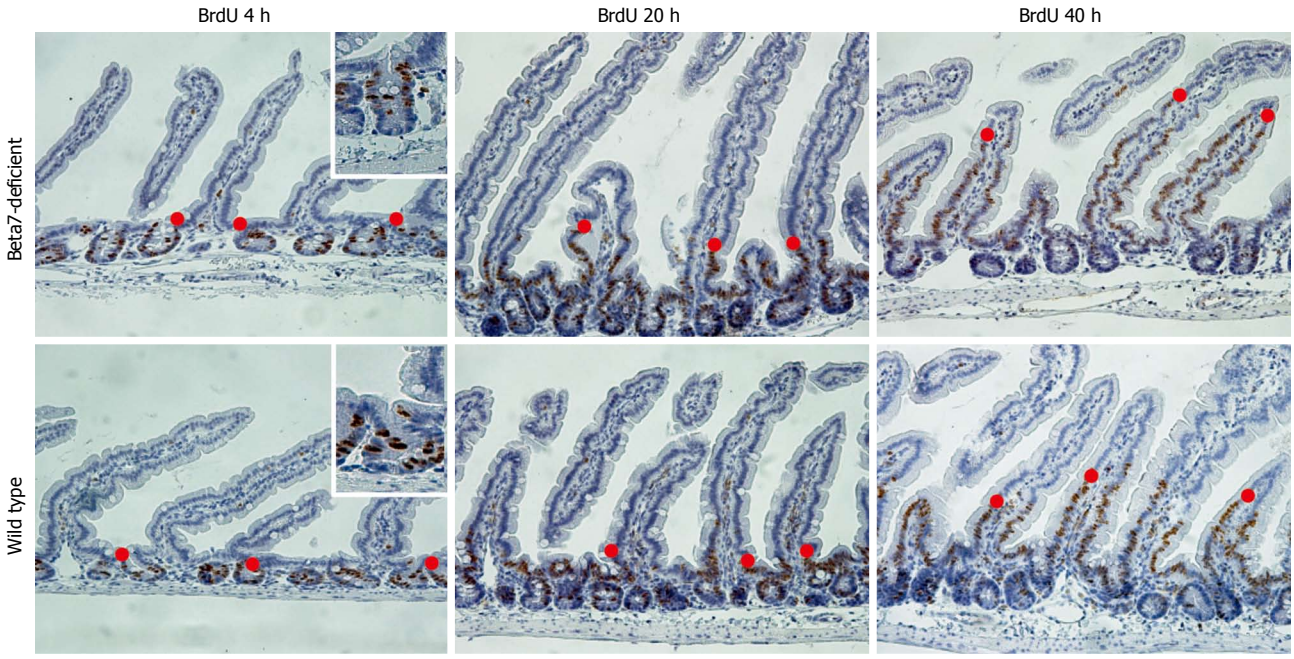

Figure 2 Morphometrical approach for evaluation of enterocyte migration. Formalin-fixed and paraffin-embedded small intestinal tissues of beta-7 integrin-deficient mice or wild type mice were anti-BrdU immunostained. BrdU was injected 4, 20, or 40 h before sacrificing mice. Red dots illustrate examples of cell^{max} location. The insets at 4 h illustrate staining results. Original magnification, × 200.

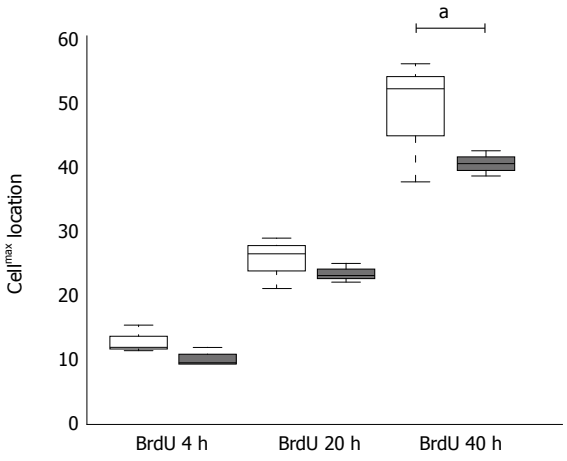

Figure 3 Enterocyte migration is accelerated in beta-7 integrin-deficient mice. Cell^{max} locations summarized from duodenum, jejunum, and ileum of beta-7 integrin-deficient mice (white) and wild type littermates (black) 4, 20, 40 h after BrdU application (three animals per group). $^{a}P < 0.05$ between group.

identical in all small intestinal segments investigated, *i.e.,* duodenum, jejunum, and ileum.

DISCUSSION

Cellular migration is a fundamental phenomenon which is determined by a plethora of molecules and regulated by diverse molecular pathways[10]. In the intestine, migration of epithelial and non-epithelial cells is essential for integrity as well as maintenance of the intestinal mucosal barrier and important to establish intestinal immunity[19-21].

Integrins are important players in establishment of cellular migration[8,13]. Integrins containing the beta-7 subunit are exclusively expressed on leukocytes and important for cell trafficking as well as homing and retention of lymphocytes into the intestinal mucosa[13,15,22]. It has been shown that beta-7 integrins bind MAdCAM-1, which is found on endothelial cells in small venules, and E-cadherin, which is strongly expressed by enterocytes[23].

In the present study we evaluated the influence of a loss of beta-7 integrin, which is essential for homing and retention of lymphocytes within the mucosal epithelial layer, on the continuous migration of enterocytes. The study was focussed on the small intestine where configuration of the GALT is more essential than in the colorectum.

Our study gives evidence that both establishment of the GALT and enterocyte migration are affected in the small intestinal mucosa of beta-7 integrin-deficient mice. The finding that these animals display diminished numbers of IELs is in line with several previous studies[13,15,23]. The finding that the injured configuration of IELs in the mucosa of beta-7 integrin knockout mice is additionally associated with a significant increase in enterocyte migration, which is likely secondary to the lymphocyte homing defect, is new.

In view with the literature, the E-cadherin beta-7 integrin pathway could be a molecular basis for an increased enterocyte migration in beta-7 integrin knockout mice[23]. Binding of lymphocytes to epithelial cells is strongly mediated by a molecular link between alphaE beta-7 integrin and E-cadherin[16]. In addition, E-cadherin - the major constituent of adherens

junctions - is preferentially involved in the configuration of apical junctional complexes and a major prerequisite for cell as well as tissue polarization. Ultra-structurally, E-cadherin binds intracellular to catenins (*e.g.,* beta-catenin, p120, and plakoglobin) which link E-cadherin activities to the actin cytoskeleton[24]. Disturbed E-cadherin function has been linked to diverse pathological processes and diseases involving an injured intestinal homeostasis and barrier function[25,26].

A crucial role of E-cadherin in migration of enterocytes was recently shown[27]. Using the BrdU technique they demonstrated that loss of E-cadherin was associated with a significant increase in cell migration and development of cell death. In view with these data, epithelial cell migration within the intestinal mucosa is probably modified by several mechanisms including the composition of epithelial and non-epithelial cell types and the integrity of E-cadherin/adherens junctions. In a putative dynamic model, epithelial migration at the intestinal surface is probably lower in heterologue systems (epithelium with normal or increased IELs; *e.g.,* wild type mice) than in homologue systems (epithelium without or low IELs; *e.g.,* beta-7 integrin knockout mice) or adherens junction-defect systems (loss of E-cadherin/catenins; *e.g.,* conditionally inactivated *Cdh1* gene in intestinal epithelia). This point of view is further substantiated by the observation that with an increasing content of goblet cells within the surface epithelial layer (heterologue system) enterocyte migration diminishes from the small intestine (turnover time 48.3 h) to colon (83.5 h) and rectum (101.2 h)[2]. The present study does not rule out the possibility that other molecular mechanisms than a disturbed beta-7 integrin-mediated enterocyte - lymphocyte communication exists determining the migration defects. However, further studies are necessary to investigate the impact of cellular composition within the intestinal epithelial layer to enterocyte migration and its putative role in pathophysiological circumstances including inflammatory and non-inflammatory intestinal disorders.

In conclusion, our study demonstrates that beta-7 integrin is not only involved in regulating homing and retention of lymphocytes but also in regulating migration of enterocytes within the small intestine surface lining epithelium.

COMMENTS

Background

Beta-7 integrins determine the migration of lymphocytes into the lamina propria and facilitate formation of the gut-associated lymphoid tissue. AlphaE beta-7 integrin mediates the retention of lymphocytes in the gut epithelium *via* E-cadherin. The intercellular protein is strongly synthesized by enterocytes.

Research frontiers

A significant reduction of intra-epithelial lymphocytes, lamina propria lymphocytes, hypoplasia of Peyer's patches, and a reduced number of IgA- and IgM-producing cells are found in the small intestine of beta-7 integrin-deficient mice. In this study, the authors demonstrate that beta-7 integrin deficiency probably affects enterocyte migration.

Innovations and breakthroughs

Recent reports have highlighted the importance of beta-7 integrins in configuration the gut-associated lymphoid tissue and lymphocyte migration. This is the first study demonstrating that beta-7 integrin deficiency is associated with a disturbed enterocyte migration. Loss of beta-7 integrin accelerates enterocyte migration along the crypt-villus axis probably mediated *via* the E-cadherin beta-7 integrin pathway.

Applications

The observation that beta-7 integrin affects enterocyte migration could be crucial for the better understanding how immunological mechanisms modulate intestinal barrier configuration.

Terminology

Integrins are proteins involved in cell-cell as well as cell-matrix cross talks. They are found in several classes and isoforms. Beta-7 integrins are exclusively expressed on leukocytes and important for cell trafficking as well as homing and retention of lymphocytes.

Peer-review

In this study, the authors attempt to demonstrate that beta-7 integrin controls enterocyte migration within the small intestinal surface lining epithelial layer. They have used beta-7 integrin deficient mice for this study.

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ORIGINAL ARTICLE

Basic Study

Sirtuin 1 in rat orthotopic liver transplantation: An IGL-1 preservation solution approach

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Author contributions: Pantazi E and Zaouali MA designed and performed the experimental work; Pantazi E, Zaouali MA, Bejaoui M and Folch-Puy E provided protocols and analysed data; Zaouali MA and Bejaoui M established the animal experimental model; Varela AT, Rolo AP and Palmeira CM determined NAD+, NAMPT levels; Ben Abdennebi H, Palmeira CM and Roselló-Catafau J contributed to the critical analyses of the data; Pantazi E, Zaouali MA, Folch-Puy E and Roselló-Catafau J coordinated the experiments and wrote the paper; all authors have read and approved the final manuscript.
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Abstract

AIM: To investigate the possible involvement of Sirtuin 1 (SIRT1) in rat orthotopic liver transplantation (OLT), when Institute Georges Lopez 1 (IGL-1) preservation solution is enriched with trimetazidine (TMZ).

METHODS: Male Sprague-Dawley rats were used as donors and recipients. Livers were stored in IGL-1 preservation solution for 8h at 4 ℃, and then underwent OLT according to Kamada's cuff technique without arterialization. In another group, livers were stored in IGL-1 preservation solution supplemented with TMZ, at 10^{-6} mol/L, for 8 h at 4 ℃ and then underwent OLT. Rats were sacrificed 24 h after reperfusion, and liver and plasma samples were collected. Liver injury (transaminase levels), mitochondrial damage (glutamate dehydrogenase activity) oxidative stress (malondialdehyde levels), and nicotinamide adenine dinucleotide (NAD^+), the co-factor necessary for SIRT1 activity, were determined by biochemical methods. SIRT1 and its substrates (ac-FoxO1, ac-p53), the precursor of NAD^+, nicotinamide phosphoribosyltransferase (NAMPT), as well as the phosphorylation of adenosine monophosphate activated protein kinase (AMPK), p-mTOR, p-p70S6K (direct substrate of mTOR), autophagy parameters (beclin-1, LC3B) and MAP kinases (p-p38 and p-ERK) were determined by Western blot.

RESULTS: Liver grafts preserved in IGL-1 solution enriched with TMZ presented reduced liver injury and mitochondrial damage compared with those preserved

in IGL-1 solution alone. In addition, livers preserved in IGL-1 + TMZ presented reduced levels of oxidative stress. This was consistent with enhanced SIRT1 protein expression and elevated SIRT1 activity, as indicated by decreased acetylation of p53 and FoxO1. The elevated SIRT1 activity in presence of TMZ can be attributed to the enhanced NAMPT protein and NAD^+/NADH levels. Up-regulation of SIRT1 was consistent with activation of AMPK and inhibition of phosphorylation of mTOR and its direct substrate (p-p70S6K). As a consequence, autophagy mediators (beclin-1 and LC3B) were over-expressed. Furthermore, MAP kinases were regulated in livers preserved with IGL-1 + TMZ, as they were characterized by enhanced p-ERK and decreased p-p38 protein expression.

CONCLUSION: Our study shows that IGL-1 preservation solution enriched with TMZ protects liver grafts from the IRI associated with OLT, through SIRT1 up-regulation.

Key words: Sirtuin 1; Ischemia-reperfusion injury; Liver transplantation; IGL-1 preservation solution; Trimetazidine

Core tip: Sirtuin 1 (SIRT1) has been implicated in pathways associated with ischemia-reperfusion injury (IRI), but its role in rat orthotopic liver transplantation has not yet been established. In our study, SIRT1 protein expression levels and activity increased when Institut Georges Lopez 1 (IGL-1) preservation solution was supplemented with trimetazidine, which was associated with less hepatic injury and mitochondrial damage. The increased deacetylation of FoxO1 by SIRT1 agreed with less oxidative stress and the activation of the autophagy pathway. These findings support the notion that SIRT1 up-regulation may be an effective strategy for reducing IRI and improving liver transplantation outcome.

Pantazi E, Zaouali MA, Bejaoui M, Folch-Puy E, Ben Abdennebi H, Varela AT, Rolo AP, Palmeira CM, Roselló-Catafau J. Sirtuin 1 in rat orthotopic liver transplantation: An IGL-1 preservation solution approach. *World J Gastroenterol* 2015; 21(6): 1765-1774 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i6/1765.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i6.1765

INTRODUCTION

Liver ischemia-reperfusion injury (IRI) can cause primary graft non-function and may lead to organ failure[1,2]. The oxygen deprivation during ischemia provokes depletion of cellular energy, whereas the subsequent re-oxygenation during reperfusion initiates a cascade of complex pathways, including the production of reactive oxygen species (ROS), which in part are responsible for the subsequent induction of hepatocellular injury. Given the complexity of IRI pathophysiology, a more profound knowledge of the underlying mechanisms is needed in order to design new therapeutic strategies able to minimize its adverse effects.

SIRT1 is a histone deacetylase that either activates or suppresses the transcription activities of various non-histone proteins through its nicotinamide adenine dinucleotide (NAD^+)-dependent activity. SIRT1 has been associated with the pathophysiology of IRI in several organs[3]. In fact, SIRT1 is involved in a wide variety of cellular processes, including apoptosis, cellular stress and autophagy[4-7]. It has been reported that SIRT1 deacetylates p53, thus reducing its transcriptional activity and its ability to induce apoptosis[8]. Forkhead box-containing protein O 1 (FoxO1) is also a target for SIRT1, and its deacetylation has been implicated in the detoxification of ROS and the promotion of autophagy[9]. Furthermore, we have recently shown that SIRT1 activation contributes, in part, to the protective effects of liver ischemic preconditioning against IRI[10].

Adequate liver preservation is vital for the success of transplantation, in order to maintain graft quality after cold storage. At present, University of Wisconsin (UW) solution is the most widely used preservation solution. Recent studies have demonstrated that Institute Georges Lopez 1 (IGL-1) preservation solution is a valuable alternative for liver grafts in orthotopic liver transplantation (OLT)[11,12]. Moreover, supplementation of IGL-1 with trimetazidine (TMZ) has been shown to increase the preservation of both steatotic and non-steatotic liver grafts in an isolated and perfused "*ex vivo*" model[13]. However, the role of SIRT1 in rat OLT when IGL-1 solutions are used has not been assessed to date.

Given that TMZ is a promising additive for increasing liver graft preservation and since SIRT1 exerts a protective role against warm IRI in the liver, the aim of this study is to investigate the potential role of SIRT1 in rat OLT when TMZ-enriched IGL-1 preservation solution is used.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (200-250 g) were used as donors and recipients. Throughout the study, animals were housed in conventional animal facilities where temperature and humidity were controlled with a 12 h light/dark cycle. All animals had free access to water and a standard laboratory diet. All procedures were performed under isofluorane inhalation anesthesia. The experiments were approved by the Ethics Committees for Animal Experimentation (CEEA, Directive 400/12), University of Barcelona and all procedures complied with European Union regulations for animal experiments (EU guideline 86/609/EEC). Rats were randomly distributed into groups as de-

scribed below.

Experimental design

The following experimental groups were created: (1) Sham (n = 6): Animals underwent transverse laparotomy and received silk ligatures in the right suprarenal vein, diaphragmatic vein, and hepatic artery; (2) IGL-1 (n = 6): Livers were flushed and stored in IGL-1 preservation solution for 8h at 4 ℃, and then underwent OLT according to Kamada's cuff technique without arterialization. Rats were sacrificed 24 h after reperfusion for liver and plasma sample collection; and (3) IGL-1 + TMZ (n = 6): Same as group 2, but livers were preserved in IGL-1 solution supplemented with trimetazidine (TMZ) at 10^{-6} mol/L.

Transaminase assay

Hepatic injury was assessed in terms of alanine aminotransferase (ALT) levels with commercial kits from RAL (Barcelona, Spain). Briefly, plasma extracts were collected before liver extraction and centrifuged at 4 ℃ for 10 min at 0.8 g. Then, 200 μL of the supernatant was added to the substrate provided by the commercial kit. ALT levels were determined at 365 nm with a UV spectrometer (DU 800, Beckman Coulter) and calculated following the supplier's instructions[14].

Glutamate dehydrogenase activity

Glutamate dehydrogenase (GLDH) is a mitochondrial enzyme that catalyses the conversion of glutamate to 2-oxoglutarate. It was used as an indirect marker of mitochondrial damage; it was measured in plasma, as described previously[15].

Lipid peroxidation assay

Lipid peroxidation in the liver was used as an indirect measure of the oxidative injury induced by ROS. Lipid peroxidation was determined by measuring the formation of malondialdehyde (MDA) with the thiobarbiturate reaction[16]. Liver samples were homogenized in Tris-HCL pH = 7 and 250 μL of trichloroacetic acid (TCA) were added to 250 μL of liver homogenates. Then, the samples were centrifugated in 3000 rpm at 4 ℃ for 15 min. Then, 250 μL of thiobarbituric acid (TBA) were added to the supernatant and were heated at 100 ℃ for 30 min. MDA reacted with TBA to form a pink chromogenic compound whose absorbance at 540 nm was measured. The result was expressed as nmols/mg protein.

NAD^+/NADH determination

NAD^+/NADH from liver were quantified with a commercially available kit (MAK037, Sigma Chemical, St. Louis, MO, United States) according to the manufacturer's instructions.

Western blot analysis

Liver tissue was homogenized in HEPES buffer as previously described[10]. Fifty μg of proteins was separated on 8%-15% SDS-PAGE gels and transblotted on PVDF membranes (Bio-Rad). Membranes were then blocked for one hour with 5% (w/v) non-fat milk in T-TBS and incubated overnight at 4 ℃ with antibody against SIRT1 (#07-131), p-mTOR (Ser2481, #09-343), mTOR (#04-385), all purchased from Merck Millipore, Billerica, MA; ac-p53 (ab37318, abcam, United kingdom); ac-FoxO1 (D-19, sc-49437), BECN1 (H-300, sc-11427), both purchased from Santa Cruz Biotechnology Inc, CA, United States; p-AMPK (Thr172, #2535), p-p38 mitogen activated protein (MAP) kinase (Thr180/Tyr182,#9211), p-70S6K (Thr389, #9205), LC3B (#2775), p-p44/42 MAPK (Erk1/2, Thr202/Tyr204), #9101, all from Cell Signaling, Danvers, MA, NAMPT (AP22021SU, Acris Antibodies GmbH, Germany), HSP70 (610607, Transduction Laboratories, Lexington, KY) and b-actin (A5316, Sigma Chemical, St. Louis, MO, United States). Membranes were then incubated for 1 h at room temperature with the corresponding secondary antibody linked to horseradish peroxidase. Bound complexes were detected using WesternBright ECL-HRP substrate (Advansta, Barcelona, Spain) and were quantified using the Quantity One software for image analysis. Results were expressed as the densitometric ratio between the protein of interest and the loading control (b-actin).

Statistical analysis

Data are expressed as mean ± SE. Statistical comparison was performed by variance analysis, followed by the Student-Newman-Keuls test (Graft Pad prism software). $P < 0.05$ was considered significant.

RESULTS

Liver injury and mitochondrial damage

We first determined liver and mitochondrial injury through transaminase and GLDH levels respectively in the different experimental groups. Livers preserved in IGL-1 and subjected to OLT showed the highest ALT and GLDH levels, whereas addition of TMZ to the IGL-1 solution resulted in a significant decrease in liver and mitochondrial injury in comparison with IGL-1 solution alone (Figure 1).

SIRT1, NAMPT, ac-p53 ac-FoxO1 proteins expression and NAD^+ levels

In order to explore the potential involvement of SIRT1 in the protective effects of TMZ on rat OLT, we first determined its protein expression pattern. SIRT1 protein expression was significantly increased when livers were preserved in IGL-1 solution compared with the Sham group (Figure 2C). Interestingly, the addition of TMZ to IGL-1 solution clearly enhanced SIRT1 expression compared with IGL-1 solution alone (Figure 2C). In view of the altered SIRT1 protein levels, we then investigated parameters associated with SIRT1 activity, such as the protein expression of

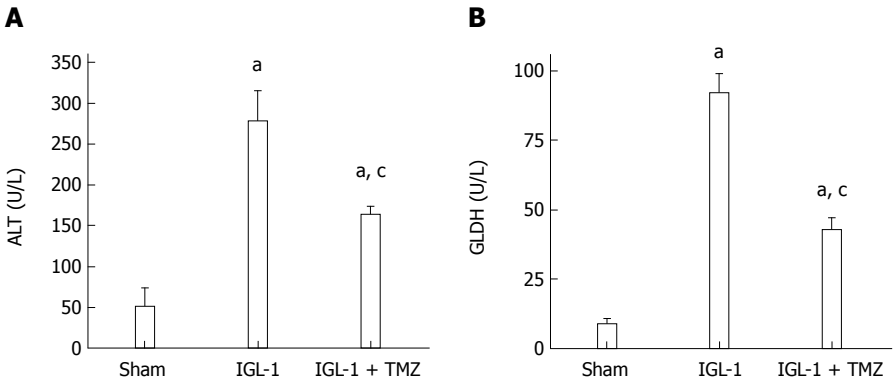

Figure 1 Alanine aminotransferase levels (A) and hepatic glutamate dehydrogenase (B) in plasma after 24 h of reperfusion. Sham: Liver harvested without transplantation; IGL-1: Liver transplanted after 8 h of cold storage in IGL-1 solution; IGL-1 + TMZ: Liver transplanted after 8 h of cold storage in IGL-1 solution with 10-6 M Trimetazidine (TMZ). [a]*P* < 0.05 *vs* Sham; [c]*P* < 0.05 *vs* IGL-1. ALT: Alanine aminotransferase; GLDH: Glutamate dehydrogenase.

nicotinamide phosphoribosyltransferase (NAMPT), the NAD^+/NADH levels, as well as the acetylation state of two direct substrates, p53 and FoxO1. As shown in Figure 2A, liver graft preservation in IGL-1 solution led to high NAMPT protein expression, which was further enhanced in case of IGL-1 supplemented with TMZ. Furthermore, NAD^+/NADH levels were significantly reduced in both IGL-1 groups in comparison to non-treated animals (Figure 2B). However, the presence of TMZ in IGL-1 resulted in a better preservation of NAD^+/NADH levels than the IGL-1 alone (Figure 2B). This was consistent with decreased acetylated FoxO1 and p53 protein levels in IGL-1 + TMZ group compared with IGL-1 (Figure 2D and Figure 2E respectively). These results suggest an increase in SIRT1 activity in the IGL-1 + TMZ group; therefore, the protective effect of TMZ is exerted, at least in part, through the induction of both SIRT1 expression and activation.

Oxidative stress and HSP70 protein expression

Next, we evaluated lipid peroxidation as an indicator of oxidative stress in OLT. Livers preserved in IGL-1 solution showed significantly increased MDA compared with Sham (Figure 3A). This increase was prevented by the addition of TMZ in IGL-1 solution. Moreover, livers preserved in IGL-1 solution up-regulated the levels of the cytoprotective heat shock protein 70 (HSP70), which was further enhanced in the presence of TMZ (Figure 3B).

p-AMPK and p-mTOR activation

It is known that both AMP-activated protein kinase (AMPK) and SIRT1 regulate each other and share many common target molecules[17]. We therefore assessed the possible involvement of AMPK activation in the protective effects of TMZ. As shown in Figure 4A, livers preserved with IGL-1 solution showed increased phosphorylated AMPK (p-AMPK). However, this AMPK activation was further enhanced in the presence of TMZ. Given that the mammalian target of rapamycin (mTOR) activity is regulated by AMPK, we next evaluated both phosphorylated mTOR (p-mTOR) and mTOR protein levels as well as the phosphorylation levels of its direct substrate, p-p70S6K. As shown in Figure 4B and C, the addition of TMZ to IGL-1 solution significantly reduced p-mTOR/mTOR and p-p70S6k protein levels compared with IGL-1 preservation solution alone.

Autophagy: Beclin-1 and LC3B protein levels

Autophagy is a conserved cellular process that is activated under conditions of nutrient stress to promote cell survival. Given the importance of the mTOR signaling pathway in the suppression of autophagy[18] and the fact that IGL-1 preservation solution enriched with TMZ was characterized by inactivation of both mTOR and p70S6k protein, we sought to test whether TMZ-induced SIRT1 protective effects might be mediated through the activation of autophagy. To do so, we explored the expression of beclin-1 and LC3B, two well-known proteins involved in the autophagic pathway. Both proteins presented significant up-regulation in the IGL-1 + TMZ group compared with both Sham and IGL-1 group (Figure 5A, B).

MAP kinases

Finally, we explored the modulation of MAP kinases by TMZ. Here we observed increased phosphorylation of extracellular signal-regulated kinase (ERK) in IGL-1 preserved livers, which was further enhanced when TMZ was added to the preservation solution (Figure 6A). Moreover, the presence of TMZ in IGL-1 solution reversed the increased phosphorylation of p38 protein levels detected in the livers preserved in IGL-1 solution (Figures 6B and 7).

DISCUSSION

The present study provides the first evidence that SIRT1 up-regulation in a rat model of OLT contributes to a better preservation of liver grafts against IRI. SIRT1 over-expression is attributed to the use of a modified IGL-1 preservation solution enriched with

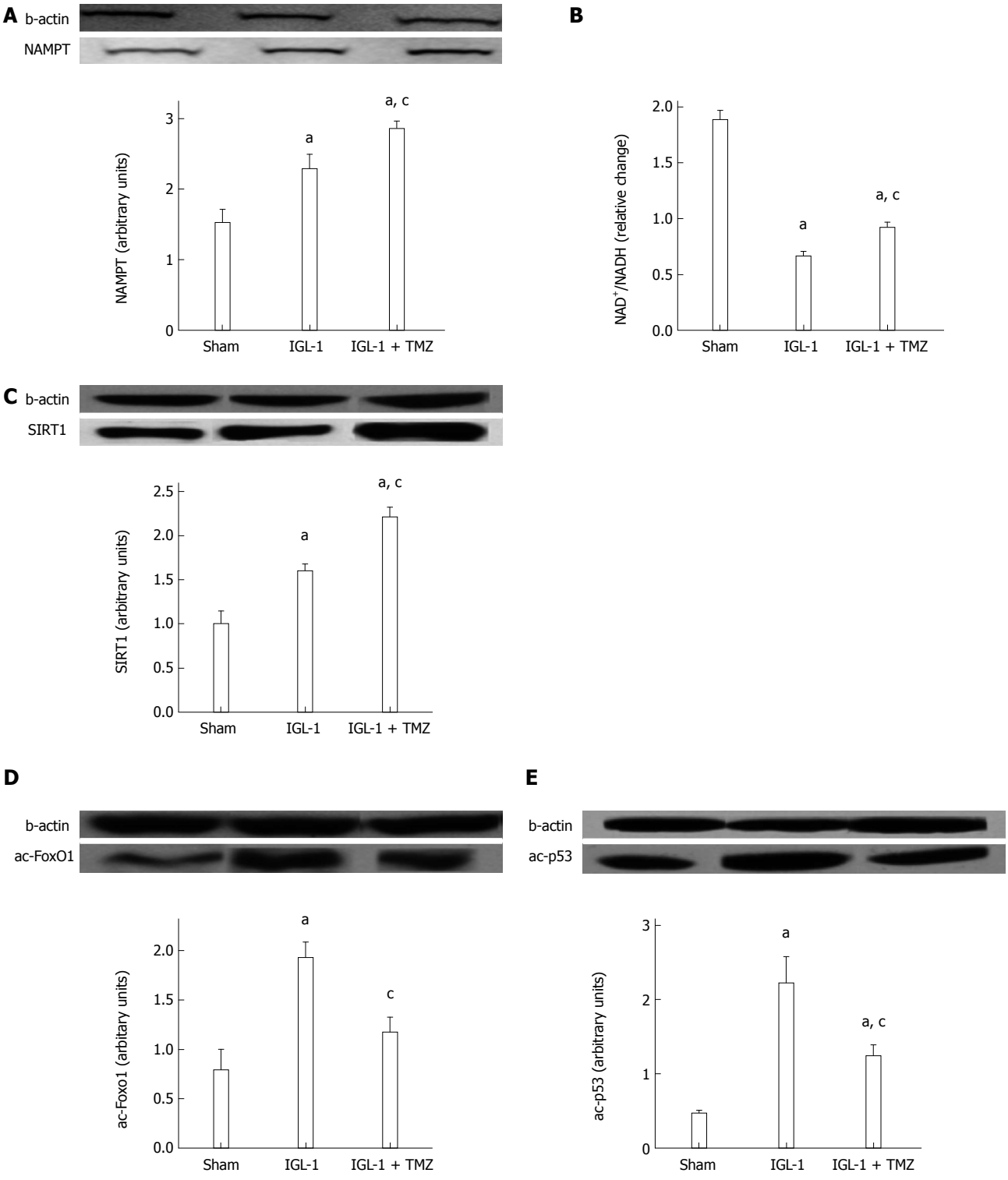

Figure 2 NAMPT protein expression (A), NAD$^+$/NADH levels (B), SIRT1 (C), ac-FoxO1 (D) and ac-p53 (E) protein expression in livers after 24 h of reperfusion. Sham: Liver harvested without transplantation, IGL-1: Liver transplanted after 8 h of cold storage in IGL-1 solution; IGL-1 + TMZ: Liver transplanted after 8 h of cold storage in IGL-1 solution with 10^{-6} mol/L trimetazidine (TMZ). $^aP < 0.05$ *vs* Sham; $^cP < 0.05$ *vs* IGL-1. NAMPT: Nicotinamide phosphoribosyltransferase; NAD$^+$: Nicotinamide adenine dinucleotide; SIRT1: Sirtuin 1; FoxO1: Forkhead box-containing protein O 1; NADH: Nicotinamide adenine dinucleotide.

TMZ, an anti-ischemic drug administered for the treatment of angina pectoris[19]. In earlier work with an isolated perfused rat liver model, we already showed that TMZ addition to IGL-1 solution promotes cytoprotective markers that are induced during ischemia, such as hypoxia inducible factor-1α through nitric oxide generation, as well as those promoted during reperfusion, such as heme oxygenase-1[13]. Here, we report that the addition of TMZ in IGL-1 preservation solution favors the activation of SIRT1 in rat OLT.

The increases observed in SIRT1 protein levels

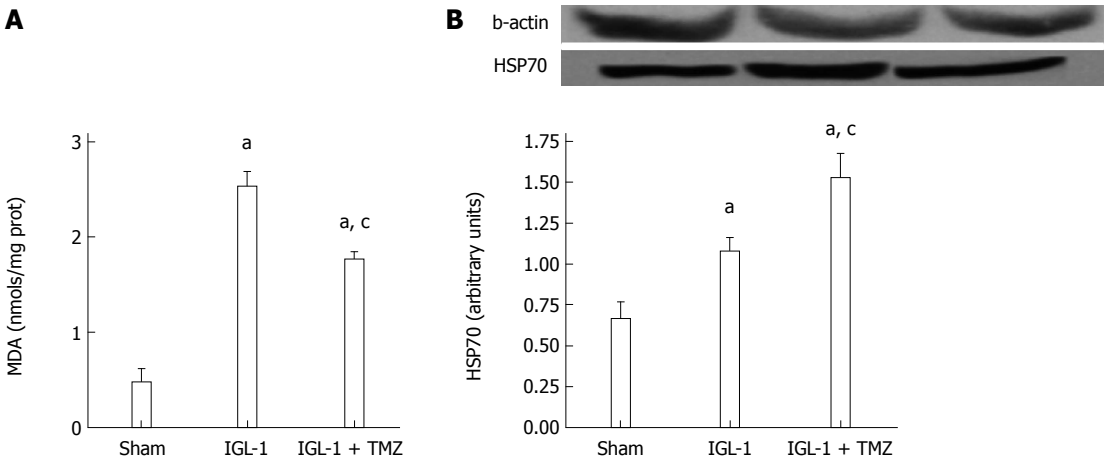

Figure 3 Malondialdehyde (A) and HSP70 protein levels (B) in livers after 24 h of reperfusion. Sham: Liver harvested without transplantation; IGL-1: Liver transplanted after 8 h of cold storage in IGL-1 solution; IGL-1 + TMZ: Liver transplanted after 8 h of cold storage in IGL-1 solution with 10^{-6} mol/L trimetazidine (TMZ). $^{a}P < 0.05$ *vs* Sham; $^{c}P < 0.05$ *vs* IGL-1. MDA: Malondialdehyde.

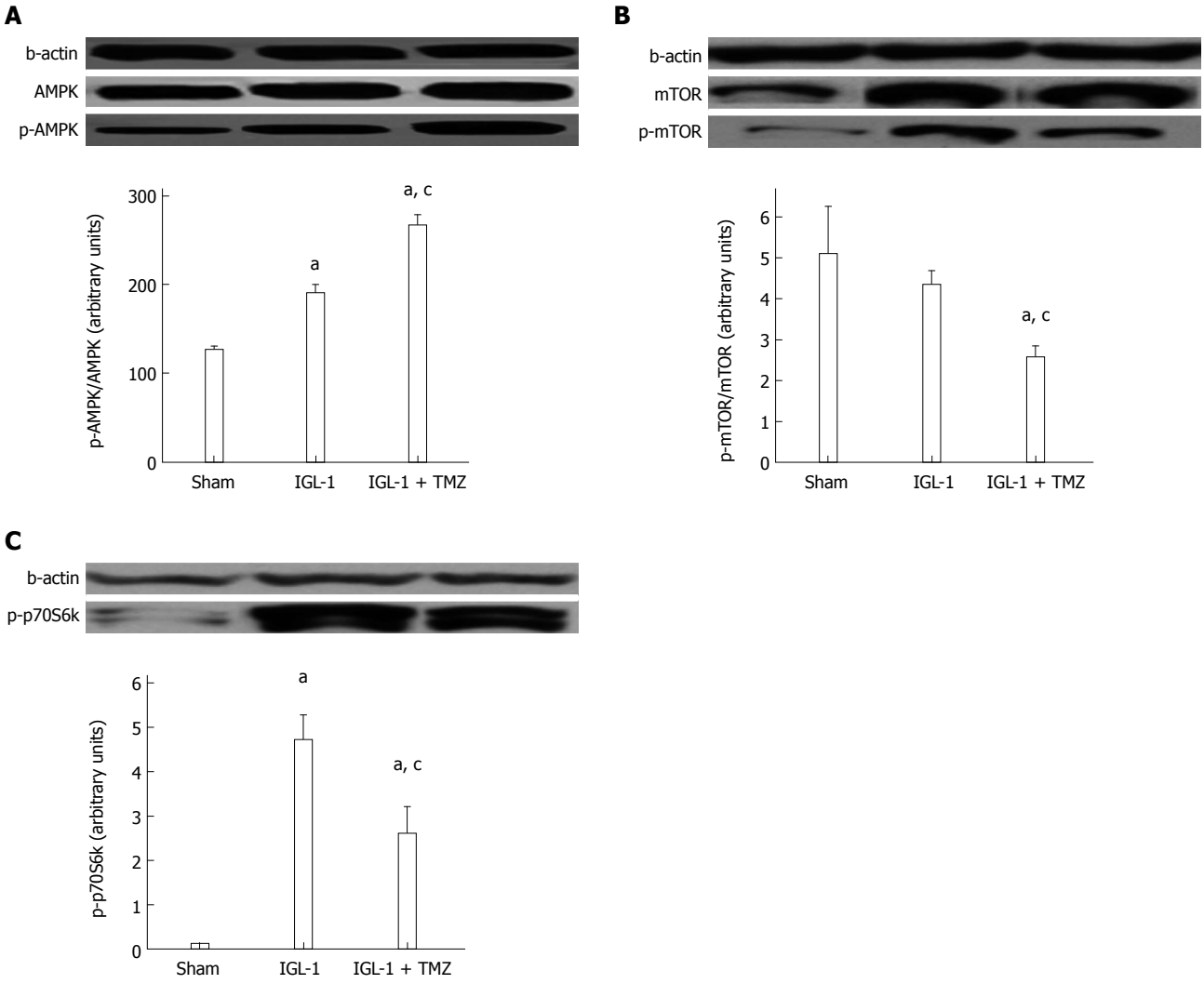

Figure 4 Protein levels of p-AMPK (A), p-mTOR/mTOR (B) and p-p70S6k (C) in livers after 24 h of reperfusion. Sham: Liver harvested without transplantation; IGL-1: Liver transplanted after 8 h of cold storage in IGL-1 solution; IGL-1 + TMZ: Liver transplanted after 8 h of cold storage in IGL-1 solution with 10^{-6} mol/L trimetazidine (TMZ). $^{a}P < 0.05$ *vs* Sham; $^{c}P < 0.05$ *vs* IGL-1.

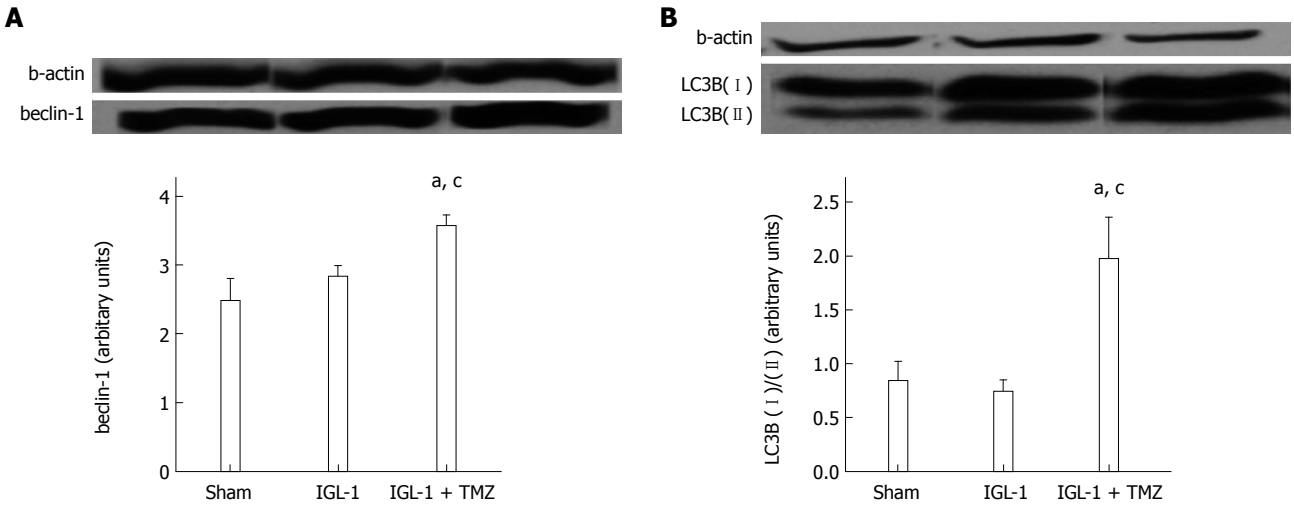

Figure 5 Protein expression of beclin-1 (A) and LC3B (Ⅱ)/(Ⅰ) (B) in livers after 24 h of reperfusion. Sham: Liver harvested without transplantation; IGL-1: Liver transplanted after 8 h of cold storage in IGL-1 solution; IGL-1 + TMZ: Liver transplanted after 8 h of cold storage in IGL-1 solution with 10^{-6} mol/L trimetazidine (TMZ). $^{a}P < 0.05$ *vs* Sham; $^{c}P < 0.05$ *vs* IGL-1.

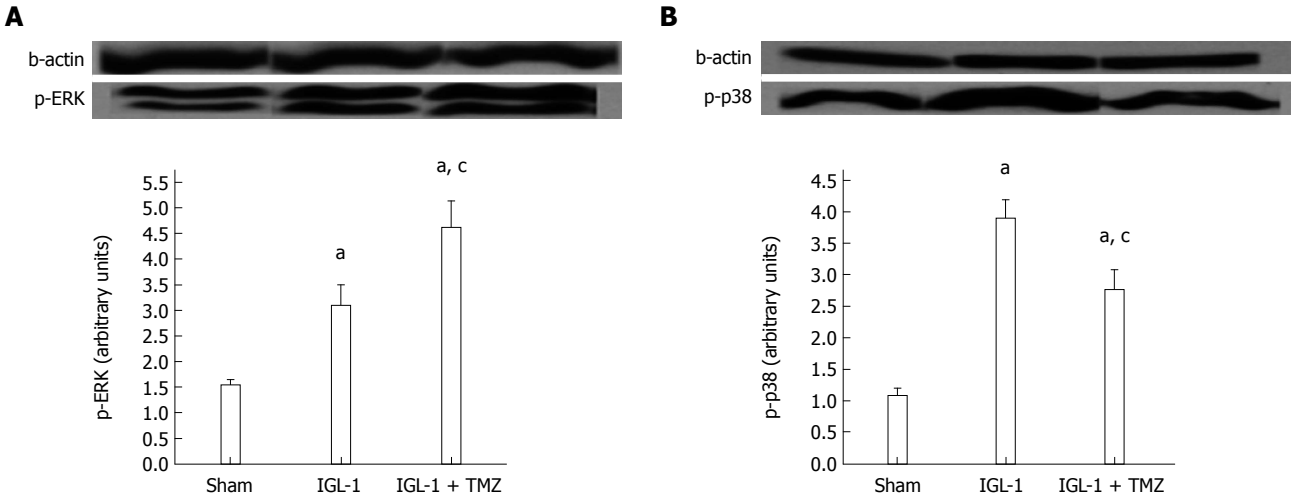

Figure 6 Protein levels of pERK (A) and p-p38 (B) in livers after 24 h of reperfusion. Sham: Liver harvested without transplantation; IGL-1: Liver transplanted after 8 h of cold storage in IGL-1 solution; IGL-1 + TMZ: Liver transplanted after 8 h of cold storage in IGL-1 solution with 10^{-6} mol/L trimetazidine (TMZ). $^{a}P < 0.05$ *vs* Sham; $^{c}P < 0.05$ *vs* IGL-1.

were consistent with an effective prevention of liver injury and mitochondrial damage. We previously reported the protective effect of TMZ on liver damage after graft preservation, and showed that its addition to UW solution reduces liver injury and improves liver function[20]. Our current data confirm that SIRT1 up-regulation in OLT due to TMZ can be considered as a protective cellular signaling response against IRI. This finding corroborates previous reports of a protective effect for SIRT1 against IRI in different organs such as heart, kidney and brain[21-23].

SIRT1 over-expression in liver grafts preserved in IGL-1 + TMZ was concomitant with increased levels of both NAMPT and NAD^+. NAD^+ is a cofactor required for SIRT1 enzymatic activity. Furthermore, it has been shown that NAMPT is the rate-limiting enzyme in the NAD^+ biosynthetic pathway and directly regulates SIRT1 activity in mammalian cells[24]. NAMPT up-regulation has been shown to be beneficial against cardiac IRI through preservation of NAD^+ levels and SIRT1 regulation[25]. Taking this into account, we suggest that in the IGL-1 + TMZ group the increased NAMPT protein levels promote NAD^+ production, which in turn contributes to the high SIRT1 deacetylase activity. Indeed, in the same group we observed decreased levels of acetylated p53 and FoxO1, two direct substrates of SIRT1, which have been reported to be SIRT1 activity markers[26,27]. Therefore, the TMZ protective effects observed are mediated, at least in part, by enhanced SIRT1 activity.

SIRT1 plays an important role in cellular stress, including the oxidative stress associated with IRI. FoxO1 deacetylation by SIRT1 has been linked with the capacity of FoxO1 to enhance the transcription

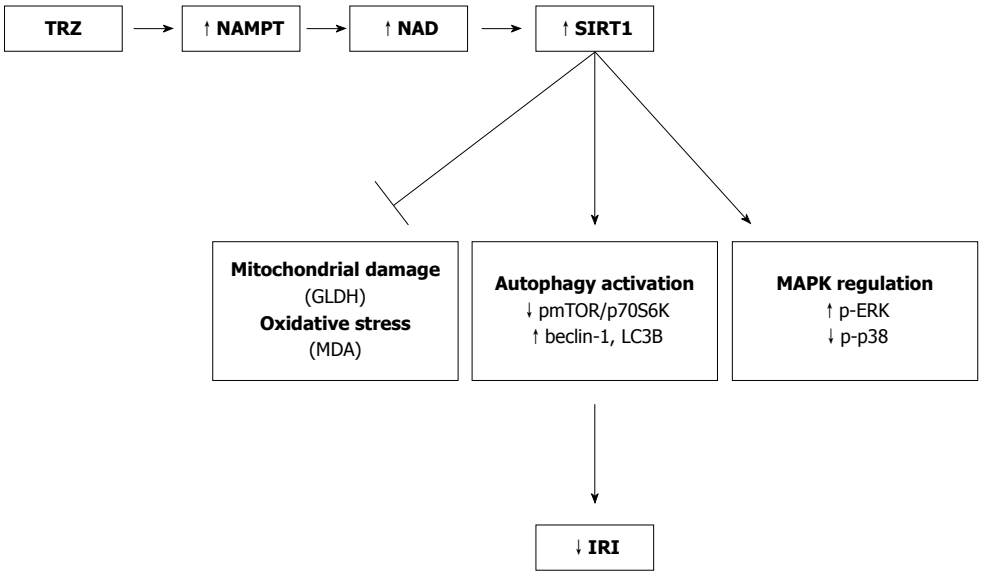

Figure 7 Schematic model of the protective effect of sirtuin 1 against ischemia-reperfusion injury associated to rat orthotopic liver transplantation. Trimetazidine (TMZ) addition to IGL-1 promotes NAMPT expression and enhances NAD+ levels, which in turn provokes SIRT1 up-regulation. SIRT1 contributes to decreased hepatic injury by inhibiting mitochondrial damage and oxidative stress, activating autophagic pathway and by enhancing p-ERK and decreasing p-p38 protein expression. SIRT1: Sirtuin 1; IRI: Ischemia-reperfusion injury; OLT: Orthotopic liver transplantation.

of anti-oxidant enzymes, thus contributing to the resistance against oxidative stress[4,5,21]. For these reasons, we decided to explore the potential relationship between SIRT1, ac-FoxO1 and the lipid peroxidation that occurs in OLT. In the present study, we found that the significant SIRT1 induction and FoxO1 deacetylation were accompanied by marked decreases in lipid peroxidation when TMZ was added to IGL-1. Moreover, SIRT1 up-regulation in OLT may contribute to reducing hepatic vulnerability against oxidative stress and to improving the mitochondrial status during oxidative stress conditions. Our results corroborate those of Ou *et al*[28] who demonstrated that over-expression of SIRT1 under oxidative stress conditions enhanced mitochondrial function in embryonic stem cells. In addition, Hsu *et al*[21] demonstrated that hearts over-expressing SIRT1 were more resistant to oxidative stress in response to IRI, due, in part, to the effective FoxO1 deacetylation by SIRT1.

It is well known that increases in heat shock protein expression are involved in the protection against oxidative stress[29]. Our results also demonstrate strong HSP70 protein expression in the IGL-1 + TMZ group. This direct connection between SIRT1 and HSP70 during liver IRI was recently reported by our group in liver ischemic preconditioning[10].

Autophagy plays an important role in both sensing oxidative stress and removing oxidative damaged proteins and organelles[30]. This process involves the inhibition of p-mTOR (mammalian TOR) and the subsequent inactivation of its direct substrate, p70S6k[31]. During autophagy, specific proteins such as beclin-1 (in the initial stages of autophagosome formation) and LC3B (during autophagosome expansion) are activated[32,33]. AMPK activation results in mTOR inhibition and subsequent autophagy activation[31]. In addition, SIRT1 deacetylase activity has been associated with activation of AMPK, and SIRT1- mediated deacetylation of FoxO1 has also been implicated in increased autophagy[34-36]. In our study, SIRT1 over-expression and the enhanced activity in the IGL-1 + TMZ group was concomitant with AMPK activation, reduced levels of p-mTOR/p-p70S6k, and increased autophagy (beclin-1 and LC3B). Our findings are in agreement with other investigations carried out in liver[37-39] and kidney[40] which have shown the protective effect of autophagy against IRI. Autophagy activation through a NAMPT/SIRT1-dependent mechanism has also been shown to be protective against cerebral ischemia[41].

However, the role of autophagy in IRI has been controversial, as various studies have evidenced either a beneficial or detrimental role. For example Jiang *et al*[40] have reported a renoprotective role of autophagy against IRI; during ischemia, autophagy can contribute to the provision of nutrients, whereas during reperfusion can eliminate damaged proteins and organelles. Similarly, Matsui *et al*[42] in cardiac IRI showed that autophagy was protective during ischemia, but beclin-1 dependent autophagy activation during reperfusion was associated with cell death. Autophagy is stimulated by various factors and it still remains unknown which steps determine the decision for the survival or death. Between them, the time of ischemia seems to influence the autophagy outcome, as a prolonged ischemia time can result in excessive activation of autophagy and subsequently to cell death[43]. In our case, autophagy is associated with decreased graft injury and thus we may suppose that eight hours of cold preservation is not sufficient

time for an excessive and detrimental activation of autophagy. More profound investigations are required in order to define the regulatory mechanisms of autophagy.

In a recent publication, we reported that SIRT1 modulates (MAP) kinases, whose activation is a consequence of oxidative stress generation associated to IRI[16]. Here, we show that TMZ addition to IGL-1 enhances p-ERK protein levels and reduces p-p38 protein levels in comparison to IGL-1 alone. As a result, the increased SIRT1 over-expression in OLT coincided with the modulation of the MAP kinases, as reported in other studies[44].

Taken together, our results show that TMZ exerts its protective role against IRI associated with OLT, in part, through the induction of SIRT1 protein expression and activity. We found that SIRT1 up-regulation prevented liver injury and oxidative stress and promoted liver autophagy (see Figure 7). Our findings support the benefits of pharmacological activation of SIRT1, a new therapeutic strategy for improving liver graft preservation.

ACKNOWLEDGMENTS

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COMMENTS

Background

Ischemia-reperfusion injury (IRI) is a complex but unavoidable situation during liver transplantation, which contributes to organ failure. Sirtuin 1 (SIRT1) is a NAD^+-dependent deacetylase that regulates several cellular pathways associated with IRI, including oxidative stress and autophagy. Institut Georges Lopez 1 (IGL-1) preservation solution has been proposed as a good alternative to UW solution for the preservation of liver grafts. Moreover, the addition of trimetazidine (TMZ), an anti-ischemic drug, to both preservation solutions has been shown to improve liver graft preservation. In this study, the authors demonstrate that TMZ addition in IGL-1 solution reduces IRI associated with rat orthotopic liver transplantation (OLT) through increases in both SIRT1 protein expression and activity.

Research frontiers

SIRT1 plays an important role in several processes, including IRI. Here we report for the first time that the presence of TMZ in IGL-1 provoked elevated FoxO1 deacetylation, oxidative stress diminution and augmented autophagy and was associated with SIRT1 activation.

Innovations and breakthroughs

SIRT1 exerts a protective effect against IRI in several organs through a variety of mechanisms. However, SIRT1 involvement in models of transplantation has not been determined to date. The present study evaluated the potential role of SIRT1 in a rat OLT model. SIRT1 was up-regulated when livers were stored in IGL + TMZ preservation solution and helped to improve the protection of liver grafts against IRI, as reflected by decreases in hepatic injury, mitochondrial damage, and oxidative stress.

Applications

Pharmacological treatment in order to enhance SIRT1 activity is a promising tool for reducing the detrimental effects of IRI associated with liver transplantation.

Peer-review

This is a relevant manuscript on to date important issue as IRI in organ transplantation as well as in other clinical condition. The manuscript is well written and the study is well conducted. The beneficial effect of adding trimetazidine on preservation solution is documented in several ways ranging from hepatic enzyme dosage to all the biological cellular expression of damage (SIRT1, ac-p53 ac-Fox O1 protein expression, to oxidative stress and HSP70 protein expression.

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ORIGINAL ARTICLE

Basic Study

Nitrite, a novel method to decrease ischemia/reperfusion injury in the rat liver

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Abstract

AIM: To investigate whether nitrite administered prior to ischemia/reperfusion (I/R) reduces liver injury.

METHODS: Thirty-six male Sprague-Dawley rats were randomized to 3 groups, including sham operated (n = 8), 45-min segmental ischemia of the left liver lobe (IR, n = 14) and ischemia/reperfusion (I/R) preceded by the administration of 480 nmol of nitrite (n = 14). Serum transaminases were measured after 4 h of reperfusion. Liver microdialysate (MD) was sampled in 30-min intervals and analyzed for glucose, lactate, pyruvate and glycerol as well as the total nitrite and nitrate (NOx). The NOx was measured in serum.

RESULTS: Aspartate aminotransferase (AST) at the end of reperfusion was higher in the IR group than in the nitrite group (40 ± 6.8 μkat/L *vs* 22 ± 2.6 μkat/L, P = 0.022). Similarly, alanine aminotransferase (ALT) was also higher in the I/R group than in the nitrite group (34 ± 6 μkat *vs* 14 ± 1.5 μkat, P = 0.0045). The NOx in MD was significantly higher in the nitrite group than in the I/R group (10.1 ± 2.9 μmol/L *vs* 3.2 ± 0.9 μmol/L, P = 0.031) after the administration of nitrite. During ischemia, the levels decreased in both groups and then increased again during reperfusion. At the end of reperfusion, there was a tendency towards a higher NOx in the I/R group than in the nitrite group (11.6 ± 0.7 μmol/L *vs* 9.2 ± 1.1 μmol/L, P = 0.067). Lactate in MD was significantly higher in the IR group than in the nitrite group (3.37 ± 0.18 mmol/L *vs* 2.8 ± 0.12 mmol/L, P = 0.01) during ischemia and the first 30 min of reperfusion. During the same period, glycerol was also higher in the IRI group than in the nitrite group (464 ± 38 μmol/L *vs* 367 ± 31 μmol/L, P = 0.049). With respect to histology, there were more signs of tissue damage in the I/R group than in the nitrite group, and 29% of the animals in the I/R group exhibited necrosis compared with none in the nitrite group. Inducible nitric oxide synthase transcription increased between early ischemia (t = 15) and the end of reperfusion in both groups.

CONCLUSION: Nitrite administered before liver ischemia in the rat liver reduces anaerobic metabolism and cell necrosis, which could be important in the clinical setting.

Key words: Ischemia-reperfusion injury; Nitrite; Liver ischemia; Liver surgery; Microdialysis; Nitric oxide; Inducible nitric oxide synthase

Core tip: Nitric oxide has an important protective effect against liver ischemia/reperfusion injury (IRI), although large levels of the substance may increase IRI. The total levels of nitrite and nitrate decrease in the liver tissue during ischemia. Because nitrite can be reduced to nitric oxide (NO) in an acidic environment, this decrease may be due to NO formation. In this study, the administration of nitrite before liver ischemia/reperfusion reduced the IRI per evaluation with transaminases, liver microdialysis and histology.

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INTRODUCTION

As liver surgery continues to evolve and expand with increasingly complicated resections being performed, vascular inflow control (Pringle's maneuver) is occasionally used to reduce bleeding associated with liver transection. Obstructing the vascular inflow to the liver inevitably leads to some degree of ischemia/reperfusion injury (IRI)[1].

It has been shown that when the availability of nitric oxide (NO) is decreased during IRI in the rat liver, there is more severe injury[2]. On the other hand, excessive NO levels, such as those found after activation of inducible nitric oxide synthase (iNOS), seem to be detrimental to the liver during IRI[3]. Although nitrite has mainly been considered an inert product of NO oxidation, nitrite can be reduced to NO in an acidic environment[4]. More recently, this process has been shown to occur, which is catalyzed by deoxyhemoglobin, at physiological oxygen levels[5]. The reduction of nitrite to NO may increase the bioavailability of NO in settings where nitric oxide synthase (NOS) does not provide NO, such as when oxygen is lacking. The administration of 48 nmol of nitrite (*via* intraperitoneal injection) before reperfusion decreases liver IRI (measured as the transaminase level at 24 h of reperfusion) in a mouse model[6]. Further studies in isolated rat mitochondria have shown that the administration of nitrite may modulate mitochondrial electron transfer in a way that slows electron transport and reduces the formation of reactive oxygen species during reperfusion[7]. This finding is consistent with the known inhibitory effects of NO on the respiratory chain[8-11]. The protective effect of nitrite on the mouse liver has further been supported by a study on the preservation of the mouse liver in University of Wisconsin solution or Lactated Ringer's solution with or without the addition of nitrite[12]. In cardiac ischemia, nitrite has been shown to decrease the infarct size, reduce apoptosis and improve contractility[13]. Inhaled NO has been investigated in human liver transplantation and shown to have some protective effect[14,15]. In a previous study, our group showed that during liver ischemia, the total level of nitrite and nitrate (NOx) is reduced in the ischemic liver tissue and that iNOS transcription is less activated during early reperfusion in rats subjected to ischemic preconditioning (IPC) than those subjected to ischemia and reperfusion alone[16].

The main hypothesis of this study was that intravenously administered nitrite before ischemia reduces IRI as measured by transaminase levels, microdialysis and histology during early (4 h) reperfusion. Additional hypotheses were that this reduction could be related to the reduced anaerobic metabolism that was detectable using microdialysis and that the treatment reduces iNOS transcription during the early phase of reperfusion.

MATERIALS AND METHODS

Study protocol and animals

The study protocol was approved by the local ethics committee for animal experiments at Linköping University, Linköping, Sweden. Thirty-six male Sprague-Dawley rats (313-444 g, Scandjur, Sollentuna, Sweden) were randomized into the following 3 groups: (1) ischemia (n = 14); (2) nitrite administered before ischemia (n = 14); and (3) sham operated (n = 8). Two minutes before a 45-min period of segmental ischemia, 480 nmol of sodium nitrite (Cayman Chemical Company; Ann Arbor, MI, United States) dissolved in saline was administered to the animals in the nitrite group by injection into the caval vein. The animals in the sham group were not subjected to ischemia, but their livers were mobilized as in the other groups. The animals were acclimatized for 1 wk at 21 ℃ on a 12-h light/dark cycle with free access to standard rat food pellets and tap water before the experiment.

Anesthesia, surgery, blood sampling and tissue sampling

The animals were anesthetized with isoflurane (Baxter Medical AB, Kista, Sweden), and 0.05 mg/kg of buprenorphine (Temgesic®, Schering-Plough, Stockholm, Sweden) was administered *sc* for pain relief. The animals were intubated with a 16 G intravenous catheter (BD Venflon, Becton, Dickinson and Company, Franklin Lakes, NJ, United States) and ventilated with a Hallowell EMC Microvent 1 respirator

(Hallowell EMC Pittsfield, MA, United States) during the experiment. The rats were monitored using a SurgiVet V3304 (Smiths Medical PM, Inc., Norwell, MA, United States), and their body temperatures were maintained within 38 ℃-39 ℃ through the use of Animaltemp 1070 heating pads (Elmedex Elektronik HB, Uppsala, Sweden). Laparotomy was performed *via* a midline incision, and the ligament attachments of the liver were divided. Immediately after intubation and at every hour throughout the experiment, the animals received 5 mL of warm (38 ℃) Ringer's acetate (Baxter Medical AB, Kista, Sweden) *sc.*

Microdialysis was carried out during the entire experiment from two separate lobes (ischemia and control) of the liver. After 15 and 40 min of ischemia and at the end of reperfusion, biopsies were taken from the ischemic lobe and stored in liquid nitrogen for later analysis. A sample of the tissue collected at the end of reperfusion was stored in formalin.

Microdialysis

CMA 20 Elite Microdialysis Probes (CMA/Microdialysis AB, Stockholm, Sweden) were inserted in both the left lateral and right lateral (control) liver lobes and perfused at a rate of 1.0 μL/min with Perfusion fluid T1 (147 mmol/L of Na, 4 mmol/L of K, 2.3 mmol/L of Ca, and 156 mmol/L of Cl at a pH of 6 and an osmolality of 290 mosm/kg) (CMA/Microdialysis AB) from a CMA 400 Syringe Pump (CMA/Microdialysis AB). Before insertion, the probes were preperfused. After inserting the probe, 35 min were allowed for the steady state to be reached, and 20 min of baseline sampling of microdialysate was performed before ischemia was induced. During ischemia, the microdialysate was sampled in 22.5-min intervals; thereafter, the intervals were 30 min. Analyses of glucose, glycerol, lactate and pyruvate were performed instantly using the clinical bedside analyzer ISCUS (CMA/Microdialysis AB).

NOx

The sum of NO_2^- and NO_3^- (NOx) was analyzed in the serum and microdialysate following the instructions provided in the commercial "Nitrite/Nitrate Fluorometric Assay Kit" (Cayman Chemical Company, Ann Arbor, MI, United States). Before analysis, the serum was ultrafiltrated through a 10-kD cut-off filter (Millipore, Solna, Sweden). The microdialysate, conversely, was directly analyzed. Standard curves were plotted. For nitrate, 10 μL of each sample was diluted in 70 μL of assay buffer. Aliquots of 10 μL of enzyme cofactor and 10 μL of nitrate reductase mixture were added to the buffered sample. After 30 min of incubation at room temperature, 10 μL of DAN (2,3-diaminonaphtalene) was added and incubated for another 10 min. For nitrite, 10 μL of each sample was diluted with 90 μL of assay buffer, and 10 μL of DAN was added. Both the nitrate and nitrite samples were then incubated for 10 min, and 20 μL of NaOH was added. All samples were read in threes with fluorometry using an excitation wavelength of 355 nm and an emission wavelength of 430 nm.

iNOS in the liver tissue

Ten milligrams of liver tissue (dry weight) was disrupted and homogenized in a Micro-Dismembranator (Braun Biotech, Allentown, PA, United States) at 2900 rpm for 30 s. RNA extraction was performed according to the manufacturer's protocol (Qiagene, Valencia, CA, United States); however, β-mercaptoethanol was not used. The RNA was eluted in 300 μL of RNAse-free water; the RNA concentration was measured at 260 nm, and its purity (approximately 2.0) was read at 260/280 nm (NanoDrop, Thermo Scientific, Erembodegem, Belgium). The RNA was further stored at -70 ℃ until assayed. Reverse transcriptase cDNA conversion with a High Capacity cDNA Reverse Transcription Kit was used on 0.5 μg RNA in a total volume of 20 μL according to the manufacturer's protocol (Applied Biosystems, Foster City CA, United States). cDNA samples were stored at -20 ℃ until assayed. Samples of 2 μL containing 50 ng cDNA (0.025 μg/μL) were used in a total reaction volume of 20 μL for the real-time PCR reaction. Samples were analyzed in triplicate with a Fast Master Mix and TaqMan Gene Expression Assay (Applied Biosystems) in a 7500 Fast Instrument (Applied Biosystems). The mean of the samples' Ct is normalized in the results against the mean Ct for glyceraldehyde-3-phosphate-dehydrogenase, which is presented as ΔCt.

Histology

Liver sections (2 μm) were hematoxylin-eosin stained and coded before examination by a pathologist who was blind to the experimental design. The degree of liver injury was estimated using a scoring system that evaluated the sinusoidal congestion (0-4), cytoplasmic vacuolation (0-4) and necrosis (0-2)[17].

Statistical analysis

The data are presented as the mean ± SE unless otherwise stated. A P value < 0.05 was considered statistically significant. *t*-tests were used to compare the treatment groups. No statistical comparisons were made between the study groups and sham group. Statistica 8.0 software (StatSoft Inc., Tulsa, OK, United States) was used for all statistical calculations.

RESULTS

Blood analyses

Liver transaminases: After 4 h of reperfusion, higher serum AST levels (Figure 1A) were found in the I/R group, 40 ± 6.8 μkat/L, compared with the nitrite group, 22 ± 2.6 μkat/L (P = 0.022). Similarly, the ALT levels (Figure 1B) at 4 h of reperfusion were

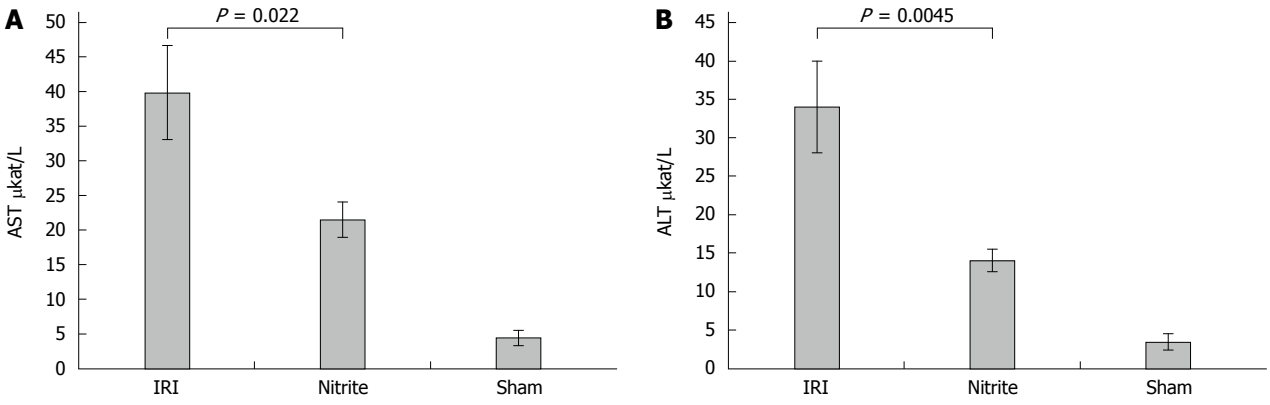

Figure 1 Serum aspartate aminotransferase and alanine aminotransferase in rats. A: The serum aspartate aminotransferase (mean ± SE) in rats subjected to 45 min of segmental (left lateral lobe) liver ischemia and 4 h of reperfusion with (nitrite, *n* = 14) or without (ischemia reperfusion injury, *n* = 14) intravenous pre-treatment with 480 nmol of nitrite and sham operated animals (*n* = 8). The animals treated with nitrite prior to ischemia and reperfusion had significantly lower aspartate aminotransferase (AST) levels (*P* = 0.022); B: The serum alanine aminotransferase (ALT) (mean ± SE) in rats subjected to 45 min of segmental (left lateral lobe) liver ischemia and 4 h of reperfusion with (nitrite, *n* = 14) or without (ischemia reperfusion injury, *n* = 14) intravenous pre-treatment with 480 nmol of nitrite and sham operated animals (*n* = 8). The animals treated with nitrite prior to ischemia and reperfusion had significantly lower AST levels (*P* = 0.0045). IRI: Ischemia reperfusion injury.

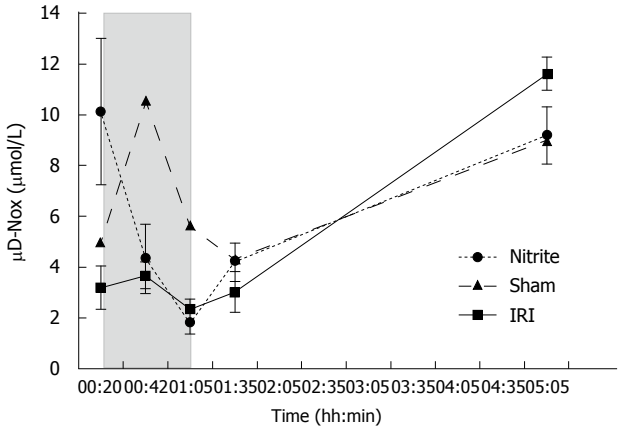

Figure 2 Parenchymal (microdialytic), nitrite and nitrate (mean ± SE) in the ischemic liver lobes in rats subjected to 45 min of segmental (left lateral lobe) liver ischemia and 4 h of reperfusion with (nitrite, *n* = 14) or without (ischemia reperfusion injury, *n* = 14) intravenous pre-treatment with 480 nmol of nitrite and sham operated animals (*n* = 8). After the administration of nitrite, the parenchymal nitrite and nitrate (NOx) was higher than for animals that did not receive nitrite (*P* = 0.031). During ischemia, the levels decreased in both groups and then increased again during reperfusion. After 4 h of reperfusion, there was a trend towards higher levels in animals that were not treated with nitrite (*P* = 0.067). The shaded area represents the ischemic phase. IRI: Ischemia reperfusion injury.

significantly higher in the IRI group, 34 ± 6 μkat/L, than in the nitrite group, 14 ± 1.5 μkat/L, (*P* = 0.0045). In the sham group, the AST and ALT levels were near normal when measured at the end of the experiment (4.4 and 3.5, respectively).

NOx: The sum of nitrite and nitrate (NOx) was measured in the serum at the end of reperfusion and was not significantly different between the groups (data not shown).

Parenchymal metabolism measured by microdialysis

NOx: After the administration of nitrite, NOx in the liver tissue was higher in the nitrite group, 10.1 ± 2.9 μmol/L, than in the I/R group, 3.2 ± 0.9 μmol/L (*P* = 0.031) (Figure 2). During ischemia, the levels of NOx decreased in both groups and thereafter increased during reperfusion. At the end of 4 h of reperfusion, there was a trend towards higher levels of NOx in the I/R group, 11.6 ± 0.7, than in the nitrite group, 9.2 ± 1.1 (*P* = 0.067).

Glucose: In both the I/R and nitrite groups, glucose increased approximately 2-fold (range: 6-12 mmol/L) in the ischemic lobe compared with either the control lobe or the pre-ischemic value in the ischemic lobe. No significant difference was found between the two ischemic lobes (Figure 3A). In the control lobes of the nitrite group, a significant (*P* < 0.001) increase (range: 6.1-7.7 mmol/L) in glucose was observed after nitrite administration. At *t* = 00:43 and during the first 95 min of the experiment, the glucose level was higher in the control lobes of the nitrite group compared with the I/R group (*P* = 0.049 and *P* = 0.02, respectively). Otherwise, there was a constant decrease in glucose during the experiment (Figure 3B).

Pyruvate: In the I/R group, pyruvate in the ischemic segments fell to approximately 40% of the level in the control segments at the end of ischemia. In the nitrite group, the corresponding value was 15%. In the early phase of reperfusion, there was a transient increase in the parenchymal pyruvate in both groups. There were, however, large interindividual variations in the pyruvate levels, and no significant differences were noted.

In the sham group, there were no significant differences between the two lobes in the microdialysate (data not shown).

Lactate: The lactate levels increased more than

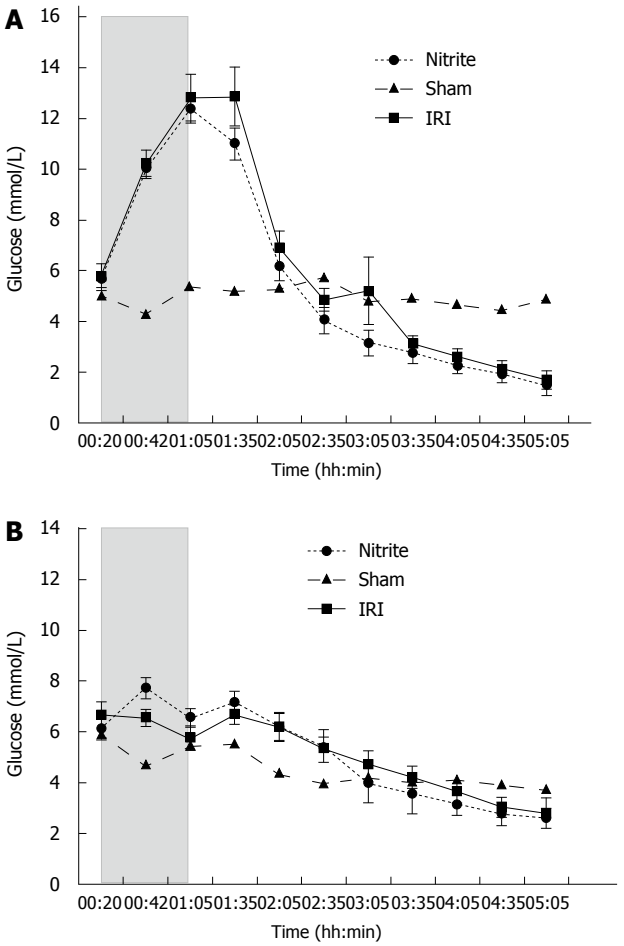

Figure 3 Parenchymal (microdialytic) glucose. A: Parenchymal (microdialytic) glucose (mean ± SE) in the ischemic liver lobes in rats subjected to 45 min of segmental (left lateral lobe) liver ischemia and 4 h of reperfusion with (nitrite, n = 14) or without (ischemia reperfusion injury, IRI, n = 14) intravenous pre-treatment with 480 nmol of nitrite and sham operated animals (n = 8). During ischemia, parenchymal glucose increased in both groups without any difference noted between the groups. In the reperfusion phase, there was a steady decline in the glucose levels. The shaded area represents the ischemic phase; B: Parenchymal (microdialytic) glucose (mean ± SE) in the control (non-ischemic, right) liver lobes in rats subjected to 45 min of segmental (left lateral lobe) liver ischemia and 4 h of reperfusion with (nitrite, n = 14) or without (IRI, n = 14) intravenous pre-treatment with 480 nmol of nitrite and sham operated animals (n = 8). After the administration of nitrite, parenchymal glucose increased significantly (P < 0.001) and reached levels higher than those in the untreated group (P = 0.02).

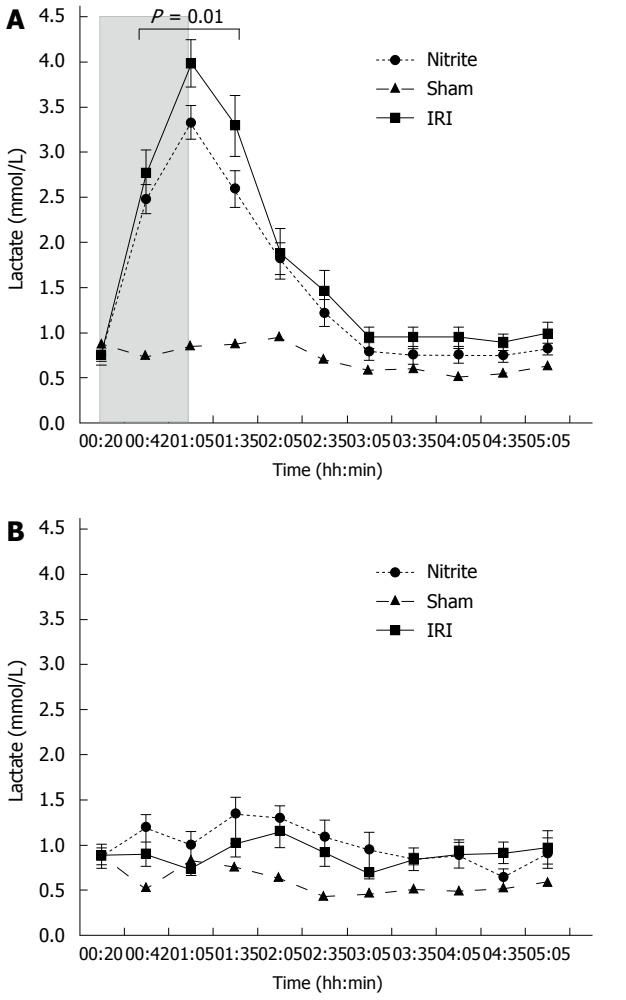

Figure 4 Parenchymal (microdialytic) lactate. A: Parenchymal (microdialytic) lactate (mean ± SE) in the ischemic liver lobes in rats subjected to 45 min of segmental (left lateral lobe) liver ischemia and 4 h of reperfusion with (nitrite, n = 14) or without (ischemia reperfusion injury, IRI, n = 14) intravenous pre-treatment with 480 nmol of nitrite and sham operated animals (n = 8). During ischemia, parenchymal lactate increased in both groups; during the ischemic phase and first 30 min of the reperfusion phase, the levels were higher in the untreated group (P = 0.01). The shaded area represents the ischemic phase; B: Parenchymal (microdialytic) lactate (mean ± SE) in the control (non-ischemic, right) liver lobes in rats subjected to 45 min of segmental (left lateral lobe) liver ischemia and 4 h of reperfusion with (nitrite, n = 14) or without (IRI, n = 14) intravenous pre-treatment with 480 nmol of nitrite and sham operated animals (n = 8). After the administration of nitrite, the parenchymal lactate increased significantly (P = 0.012) and reached levels higher than those in the untreated group (P = 0.01).

four-fold in the ischemic lobes (4 mmol/L) compared with the control lobes (0.7 mmol/L) in the I/R group during the ischemic phase; the same pattern was observed in the nitrite group (3.3 and 1 mmol/L, respectively), although the increase had a smaller in magnitude. During ischemia and the first 30 min of reperfusion, the lactate levels were significantly higher in the IRI group than in the nitrite group (P = 0.01). At the end of ischemia (t = 01:05) and after 30 min of reperfusion (t = 01:35), there was a trend towards less lactate in the nitrite group compared with the I/R group (P = 0.053 and 0.08, respectively) (Figure 4A).

Lactate increased from 0.9 to 1.2 mmol/L (P = 0.012) in the control lobes in the nitrite group after the administration of nitrite, which was not observed in the I/R group. Furthermore, the lactate levels were significantly (P = 0.01) higher during the first 95 min of the experiment in the control lobes in the animals treated with nitrite than in those subjected to I/R alone (Figure 4B).

In the sham group, the lactate levels remained low and stable throughout the experiment.

Glycerol: The increase in glycerol in the ischemic lobes (range: 18-686 μmol/L) compared with the control lobes was approximately 38-fold in the I/R

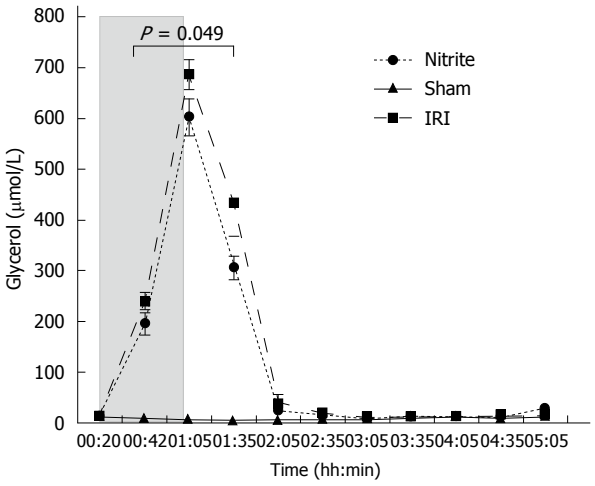

Figure 5 Parenchymal (microdialytic) glycerol (mean ± SE) in the ischemic liver lobes in rats subjected to 45 min of segmental (left lateral lobe) liver ischemia and 4 h of reperfusion with (nitrite, *n* = 14) or without (ischemia reperfusion injury, *n* = 14) intravenous pre-treatment with 480 nmol of nitrite and sham operated animals (*n* = 8). During ischemia, the parenchymal glycerol increased in both groups; during the ischemic phase and the first 30 min of the reperfusion phase, the levels were higher in the untreated group (*P* = 0.049). The shaded area represents the ischemic phase. IRI: Ischemia reperfusion injury.

group during the ischemic phase. The nitrite group followed a similar pattern, but the increase (range: 25-602 μmol/L) was less pronounced than in the I/R group (24-fold). During ischemia and the first 30 min of reperfusion, the glycerol levels were significantly higher in the IRI group than in the nitrite group (*P* = 0.049). At the end of ischemia (*t* = 01:05), there was a trend towards higher glycerol in the ischemic lobes in the I/R group compared with the nitrite group (*P* = 0.079); a similar trend was noticed after 30 min of reperfusion (*t* = 01:35, *P* = 0.069). In the ischemic lobes, the glycerol levels returned to the levels observed in the control lobes after approximately 60 min of reperfusion. No significant changes were observed in the sham group or in the control lobes (Figure 5).

Tissue analysis

iNOS mRNA: There was no difference in the iNOS mRNA between the groups 15 min into ischemia (IRI ΔCt = 9.3 ± 0.72, ΔCt nitrite = 8.6 ± 0.24). In both groups, transcription was increased at the end of the experiment to ΔCt = 3.8 ± 0.52 in the I/R group and 3.4 ± 0.26 in the nitrite group (*P* < 0.01 in both groups). There was, however, no significant difference between the groups.

Histology: The degree of liver injury was evaluated in terms of sinusoidal congestion (0-4), cytoplasmic vacuolation (0-4) and necrosis (0-2)[17].

For the sham animals, all scores were zero, except for 1 animal that scored 1 (10%-40% of hepatic tissue was affected) for sinusoidal congestion. In the

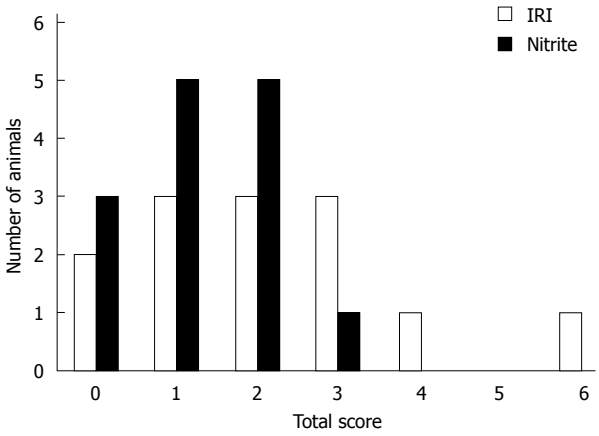

Figure 6 Distribution of total histological injury score. The distribution of total histological injury score in the ischemic liver lobes in rats subjected to 45 min of segmental (left lateral lobe) liver ischemia and 4 h of reperfusion with (nitrite, *n* = 14) or without (ischemia reperfusion injury, IRI, *n* = 14) intravenous pre-treatment with 480 nmol of nitrite.

I/R group (*n* = 14), the mean score for sinusoidal congestion was 1.29 compared with 0.86 in the nitrite group (*n* = 14). Five animals (36%) scored 2 or higher in the I/R group compared with 1 (7%) in the nitrite group. Similarly, the mean score for cytoplasmic vacuolization was 0.71 in the I/R group and 0.43 in the nitrite group, and 5 (36%) of the animals in the IRI group did not show signs of vacuolation compared with 8 (57%) in the nitrite group. Of the 14 animals in the I/R group, 4 (29%) had signs of necrosis by histology, whereas none were found in the nitrite group. Together, the median total score for the I/R group was 2 (0-6), and this value for the nitrite group was 1 (0-3); Figure 6 shows the distribution of the scores and Figure 7 shows the representative histologic slides for each group.

DISCUSSION

This is the first experimental study to investigate the effect of nitrite administered intravenously before a period of segmental liver I/R in rats. This study shows that nitrite reduces the systemic levels of AST and ALT after 4 h of reperfusion, improves anaerobic metabolism in the ischemic lobe (measured *via* microdialysis) and reduces the histological signs of IRI.

The administration of nitrite resulted in significantly higher NOx in the microdialysate before ischemia, indicating that the administered nitrite reached the liver parenchyma. The total NOx was markedly reduced in the liver parenchyma during ischemia in both groups. This reduction had previously been shown in animals that were not treated with nitrite in this model[16]. The tendency towards higher NOx in the IRI group at the end of the experiment, although not statistically significant, may reflect more endogenous NO production in the

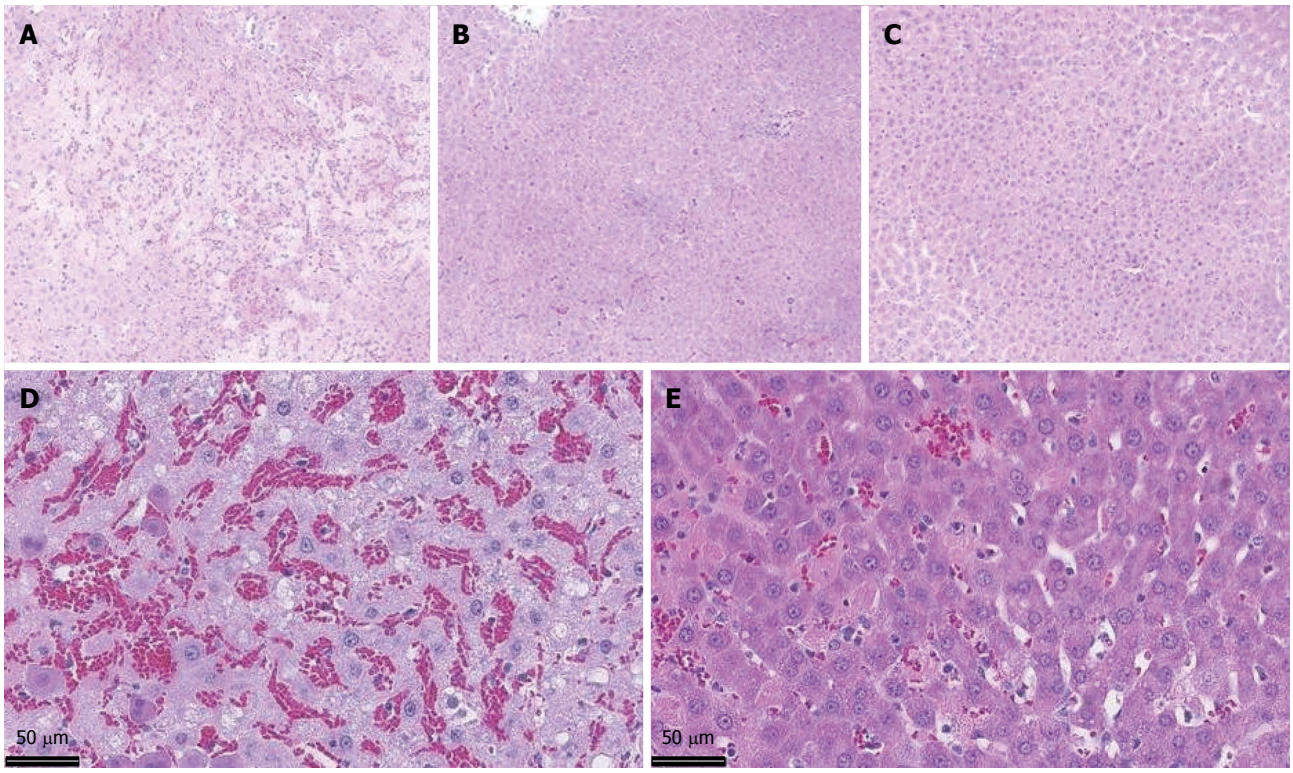

Figure 7 HE-stained liver tissue from the ischemic liver lobes in rats subjected to 45 min of segmental (left lateral lobe) ischemia and 4 h of reperfusion without pre-treatment (A), with 480 nmol of nitrite administered intravenously before ischemia (B) and sham operated (20 x magnification) (C); In 40 × magnificataion, without pre-treatment (D) and with 480 nmol of nitrite administered intravenously before ischemia (E).

I/R group during reperfusion. In the sham group, an increase in the MD NOx was noted during the experiment, which is most likely consistent with the inflammatory response to laparotomy. In the serum analysis, no differences could be found between the groups, which is consistent with our previous results and with published data from others[6,16,18]. These findings may be explained by data indicating that nitrite is converted to NO and is bound to thiols and metals[5,19]. Therefore, the administration of nitrite may increase the bioavailability of NO and, in that way, may reduce IRI.

Reduced iNOS transcription has been found to accompany reduced IRI when IPC and R-IPC are compared[20]. In the present study, no significant difference was noted, although there was a tendency towards a larger increase of transcription in the I/R group. Keeping in mind that almost 30% of the liver cells showed signs of necrosis in the I/R group and, therefore, may not have been capable of inducing transcription, this could be a false-negative finding. Another possible explanation is that the protective effects of nitrite found in this study are not related to a reduction in iNOS transcription.

In previous studies, lower glucose levels in microdialysate from the ischemic liver were associated with diminished signs of IRI[21,22]. In this study, this phenomenon was not confirmed, although the glucose levels tended to return to normal earlier in the nitrite group than they did in the I/R group (Figure 3A). Lactate was significantly higher in the ischemic lobes in the I/R group than in the nitrite group, indicating that there is reduced anaerobe metabolism when nitrite is administered. The opposite was observed in the control lobes, a finding that has not been reported in earlier IRI studies, including those using microdialysis. The increase in lactate observed in the control lobes after nitrite administration may be related to a dampening of the electron transport chain, which is in agreement with previous studies[7,8]. Dampening of the electron transport chain will decrease the oxidative stress and may therefore be important in reducing IRI. This potential explanation is further supported by the increase in hepatic glucose found in the same control lobes, which may indicate reduced metabolism. This subtle difference was not observed in the ischemic lobe, which is perhaps due to the increased anaerobic metabolism.

Glycerol was found to be significantly higher in the I/R group than in the nitrite group during ischemia and the first 30 min of reperfusion. Although the degrees of correlation with the AST (r = 0.3) and ALT (r = 0.27) levels were low and non-significant, they are consistent with studies on IRI that have included microdialysis and indicate damage to the cellular membranes[21,23]. Furthermore, the histological findings also indicate that the IRI is reduced with nitrite treatment administered before ischemia, and there is no necrosis in the treated animals, compared with almost one-third of the untreated animals.

Together, these results indicate that nitrite administered before I/R reduces the IRI, and they further corroborate an earlier study on mice that showed that nitrite administered before reperfusion reduces the IRI in a dose-dependent manner[6]. When interpreting these findings, one has to consider that the results are obtained after a short period of reperfusion; therefore, it is unclear whether the damage continues during the later phase.

Because the administration of nitrite during ischemia in the setting of liver resection would be impractical, it is important to also evaluate the effects when nitrite is administered prior to an ischemic insult, as was performed in this study. The protective effect of oral nitrite 24 h before hepatic ischemia in mice has already been established; until now, however, intravenous administration immediately prior to ischemia has not been investigated[7]. The results presented herein support that nitrite may have a protective role against IRI when administered before an ischemic period. This could have clinical implications insofar as one of the methods used to lower the central venous pressure during liver resections in humans involves the intravenous administration of nitroglycerine[24]. Nitroglycerine has a short half-life, and it is metabolized in the liver to nitrite, which may constitute a pool of substrate for NO production without NOS involvement[25]. In a small, randomized study on the effect of low central venous pressure (CVP) on bleeding during hepatectomy, nitroglycerin was among the methods used to lower the CVP. The low CVP group was had lower (although nonsignificant) ALT levels postoperatively than controls, which may be attributed to the aforementioned effects of nitrite[26].

In conclusion, the administration of nitrite before I/R reduces the biochemical and pathological signs of the IRI. This effect may have clinical implications and should be further investigated in the clinical setting.

COMMENTS

Background

When the circulation to the liver is temporarily closed (ischemia/reperfusion), as is occasionally necessary during liver surgery, there is inevitably damage to the liver. It has been shown that nitric oxide plays an important role in this process although the exact mechanisms are unknown. Furthermore, the methods to prevent this damage seem to influence the nitric oxide system.

Research frontiers

Nitrite administered into the abdomen during ischemia/reperfusion has been shown to reduce the liver damage in the mice. Furthermore, nitrite administered by the oral route in mice 24 h prior to ischemia/reperfusion has a similar effect. This novel treatment could later prove to be of value in humans, but further studies in animals are needed.

Innovations and breakthroughs

Earlier research has shown that nitrite can be reduced to nitric oxide, particularly in the acidic environment. Furthermore, nitrite and nitrate are reduced in the liver parenchyma during ischemia. This, along with findings indicating that nitrite may reduce liver damage when administered during liver ischemia/reperfusion, may indicate that pre-treatment with nitrite can prevent the damage. Until now, this has only been shown for mice. The current study supports earlier findings as the route of administration, and the animal strain differs from the previous reports.

Applications

The findings of this basic research article add to the authors' understanding of the value of nitrite as a protective treatment against damage to the liver that is related to ischemia/reperfusion. Showing that this effect occurs in rats as well as in mice (previously shown) may serve as the foundation for further research in the area. It should be noted, however, that such treatment would not immediately be adaptable to the clinical setting.

Terminology

Ischemia/reperfusion injury (IRI) is an ill-defined term that refers to the damage in the liver when the circulation is closed and then reopened. The extent of the damage is typically measured by the levels of liver transaminases in the serum.

Peer-review

In this paper, authors aim to investigate whether nitrite administration prevents liver IRI. To reach their scope, the Authors used a rat model of liver IRI. In the animal group treated with nitrates before IRI transaminases were significantly lower, as well as anaerobic metabolism markers, than in the control or the sham-operated group. Accordingly, histopathological liver tissue sections demonstrated less injury in the treated group compared to controls.

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ORIGINAL ARTICLE

Basic Study

In vitro identification of nonalcoholic fatty liver disease-related protein hnRNPM

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Abstract

AIM: To study the formation of intracellular glyceraldehyde-derived advanced glycation end products (Glycer-AGEs) in the presence of high concentrations of fructose.

METHODS: Cells of the human hepatocyte cell line Hep3B were incubated with or without fructose for five days, and the corresponding cell lysates were separated by two-dimensional gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Glycer-AGEs were detected with the anti-Glycer-AGEs antibody. Furthermore, the identification of the proteins that are modified by glyceraldehyde in the presence of high concentrations of fructose was conducted using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The protein and mRNA levels were determined by Western blotting and real-time reverse transcription PCR, respectively.

RESULTS: The results of the two-dimensional gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated a greater amount of Glycer-AGEs in the sample exposed to high concentrations of fructose than in the control. The detected Glycer-AGEs showed isoelectric points in the range of 8.0-9.0 and molecular weights in the range of 60-80 kDa. The heterogeneous nuclear ribonucleoprotein M (hnRNPM), which plays an important role in regulating gene expression by processing heterogeneous nuclear RNAs to form mature mRNAs, was identified as a modified protein using MALDI-TOF-MS. Increasing the concentration of fructose in the medium induced a concentration-dependent increase in the generated Glycer-AGEs. Furthermore, in an experiment using glyceraldehyde, which is a precursor of Glycer-AGEs, hnRNPM was found to be more easily glycated than the other proteins.

CONCLUSION: The results suggest that glyceraldehyde-modified hnRNPM alters gene expression. This change may cause adverse effects in hepatocytes and may serve as a target for therapeutic intervention.

Key words: Advanced glycation end-products; Fructose; Glycation; Glyceraldehyde; Heterogeneous nuclear

ribonucleoprotein M; Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis

Core tip: Excessive intake of fructose contributes to the development of nonalcoholic fatty liver disease and to the progression of the disease to nonalcoholic steatohepatitis. Fructose is metabolized to glyceraldehyde, which is a precursor of glyceraldehyde-derived advanced glycation end-products (Glycer-AGEs). We showed that intracellular Glycer-AGEs were formed in the presence of high concentrations of fructose. Additionally, heterogeneous nuclear ribonucleoprotein M (hnRNPM) was identified as one of the target proteins for glycation. These results suggest that the glyceraldehyde-modified hnRNPM resulting from the exposure of the cells to high concentrations of fructose, alters gene expression and causes adverse effects in hepatocytes.

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INTRODUCTION

Fructose is a major component in high fructose corn syrup (HFCS) and sucrose, which are commonly used as sweeteners in beverages and processed foods. The consumption of fructose has increased over the past 40 years, and HFCS consumption has increased rapidly to replace 50% of the sucrose consumption. HFCS is cheaper than sucrose and can be transported easily; moreover, it is more effective in stabilizing the texture of some processed foods than sucrose[1,2]. Increased fructose intake causes many adverse effects such as obesity, dyslipidemia, and insulin resistance, and contributes to the development and progression of nonalcoholic fatty liver disease (NAFLD). NAFLDs ranging from simple steatosis to steatohepatitis include the most common liver diseases worldwide[1-5].

Advanced glycation end-products (AGEs) are formed by the Maillard reaction, a nonenzymatic reaction between the ketones or aldehydes of sugars and the amino groups of proteins. AGEs are associated with aging and diabetes-related pathologic complications[6,7]. This reaction begins with the conversion of reversible Schiff base adducts to more stable covalently bound Amadori rearrangement products. Over the course of days to weeks, these Amadori products undergo further rearrangement reactions to form irreversibly bound moieties known as AGEs[8].

Recent studies have suggested that AGEs can be formed not only from sugars, but also from carbonyl compounds produced as a result of the autoxidation of sugars and from other metabolic pathways[9,10]. Evidence suggests that the glyceraldehyde-derived AGEs (Glycer-AGEs) are associated with diabetes-related pathologic complications[11-13]. Absorbed fructose is selectively metabolized in the liver and later metabolized into glyceraldehyde by aldolase[1,2]. The immunohistochemical analysis of Glycer-AGEs show intense staining in the livers of patients with nonalcoholic steatohepatitis (NASH), which is a form of NAFLD[14]. It is well known that AGE modification adversely alters protein function[15,16]. However, the effects of fructose on the formation of intracellular Glycer-AGEs remain poorly understood.

In this study, we have examined the formation of intracellular Glycer-AGEs in the human hepatocyte cell line Hep3B when exposed to high concentrations of fructose.

MATERIALS AND METHODS

Chemicals

All chemicals were commercial samples of high purity and used as supplied. Glyceraldehyde was purchased from Nakalai Tesque (Kyoto, Japan).

Cell cultures

Hep3B cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, United States) supplemented with 10% fetal bovine serum (Equitech-Bio, Kerrville, TX, United States) under standard cell culture conditions (humidified atmosphere, 5% CO_2, 37 ℃). Cells (2×10^5 cells/mL) were seeded in various plates or culture dishes (BD Biosciences, Franklin Lakes, NJ, United States) and incubated for 24 h before the start of all experiments. To form intracellular Glycer-AGEs, cells were incubated with or without 0.5-10.0 mmol/L fructose for 120 h.

Preparation of cell lysate

In the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample, cells were washed with ice-cold Ca^{2+}- and Mg^{2+}-free PBS [PBS(-)] and subjected to lysis buffer [25 mmol/L Tris-HCl (pH 7.6), 150 mmol/L sodium chloride, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, and 1× protease inhibitor cocktail (Thermo Fisher Scientific Inc., Waltham, MA, United States)]. Subsequently, cell lysates were passed through a syringe several times for further homogenization, and they were centrifuged at 12000× *g* for 10 min at 4 ℃. After the protein concentrations were measured, cell lysates were dissolved in LDS sample buffer (Invitrogen of Thermo Fisher Scientific) containing 10% sample reducing agent (Invitrogen) and boiled for 10 min at

70 ℃.

In the two-dimensional gradient SDS-PAGE sample, cells were washed with ice-cold PBS(-) and subjected to 10% trichloroacetic acid for 30 min at 4 ℃. Subsequently, collected cells were washed with PBS(-) and dissolved in urea buffer (2 mol/L thiourea, 7 mol/L urea, 3% CHAPS, 1% Triton X-100, 10% sample reducing agent) for 30 min at 4 ℃. The resulting suspension was centrifuged at 20000× *g* for 30 min at 4 ℃.

Protein concentrations were measured using the Bradford assay (Bio-Rad Laboratories Inc., Hercules, CA, United States).

Two-dimensional gradient SDS-PAGE and mass spectrometry protein identification

After measuring the protein concentrations, cell lysates that modified acrylamide (100 μg) were separated in agar gel (pH range: 3-10 or 5-10) (ATTO, Tokyo, Japan) and 5-20% SDS-polyacrylamide gradient gel (ATTO). All processes were performed according to the manufacturer's instructions.

After two-dimensional gradient SDS-PAGE, the gel was stained with EzStain Aqua (ATTO) for 3 h and washed with distilled water. The selected spots were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis. We consigned MALDI-TOF-MS analysis to Genomine, Inc (Kyungbuk, Korea).

Western blot analysis

Cell lysates were separated by electrophoresis and then electro-transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore Corporation, Billerica, MA, United States). Membranes were blocked for 60 min by using the PVDF blocking reagent for Can Get Signal (Toyobo, Osaka, Japan). After washing with PBS containing 0.05% Tween 20 (PBS-T), the membranes were incubated with rabbit anti-Glycer-AGEs antibody, mouse anti-heterogeneous nuclear ribonucleoprotein M (hnRNPM) antibody (Millipore), mouse anti-β-actin antibody (Santa Cruz Biotechnology, Dallas, TX, United States), or rabbit anti-GAPDH antibody (GeneTex, Irvine, CA, United States) in Can Get Signal Solution 1 (Toyobo) for 1 h. Subsequently, the membranes were washed three times with PBS-T and incubated with anti-rabbit IgG antibody (GeneTex) or anti-mouse IgG antibody (Dako of Agilent Technologies, Santa Clara, CA, United States) in Can Get Signal Solution 2 (Toyobo) for 1 h. After five additional washes with PBS-T, immunoreactive proteins were detected using ECL Prime Western Blotting Detection Reagents and Amersham hyperfilm ECL (GE Healthcare Ltd., Little Chalfont, Buckinghamshire, United Kingdom).

Neutralization of rabbit anti-Glycer-AGEs antibody

Glycer-AGEs were prepared as described previously[17]. Briefly, 25 mg/mL of bovine serum albumin (BSA; A0281, Sigma-Aldrich) was incubated at 37 ℃ for 7 d under sterile conditions with 0.1 mol/L glyceraldehyde and 5 mmol/L diethylenetriaminepentaacetic acid (Dojindo Laboratories, Kumamoto, Japan) in 0.2 mol/L phosphate buffer (pH 7.4). As a control, unglycated BSA was incubated under the same conditions, but without glyceraldehyde. The unglycated and glycated albumin were purified using a PD-10 column (GE Healthcare Ltd.) and dialysis against PBS. All preparations were tested for endotoxin using the Endospecy ES-20S system (Seikagaku Co., Tokyo, Japan). Protein concentrations were determined using the Dc protein assay reagent (Bio-Rad Laboratories) using BSA as a standard.

The amount of Glycer-AGEs-BSA required to neutralize rabbit anti-Glycer-AGE antibody was calculated based on a standard curve of Glycer-AGEs-BSA in a competitive ELISA. The antibody was incubated with 50 μg/mL of Glycer-AGEs-BSA for 1 h at room temperature and it was used for the subsequent experiments.

Real-time reverse transcription-PCR

Total RNA was isolated from Hep3B cells using ISOGEN Ⅱ (Nippon Gene, Tokyo, Japan), and cDNA was synthesized using random primers and reverse transcriptase. Real-time reverse transcription-PCR was performed using a Smart Cycler Ⅱ System (Takara, Shiga, Japan), as previously described[18]. The primers used were as follows: hnRNPM, 5′-GAG GCC ATG CTC CTG GG-3′ and 5′-TTT AGC ATC TTC CAT GTG AAA TCG-3′; and β-actin, 5′-TCC ACC TCC AGC AGA TGT GG-3′ and 5′-GCA TTT GCG GTG GAC GAT-3′.

Statistical analysis

All experiments were performed in duplicate and repeated at least two or three times. Each experiment yielded essentially identical results. Data are expressed as the mean ± SD. The significance of differences between group means was determined using a *t*-test. $P < 0.05$ was defined as significant.

RESULTS

Exposure to high fructose concentrations enhanced the formation of intracellular Glycer-AGEs in Hep3B cells

We examined whether intracellular Glycer-AGEs were formed by exposure to high fructose concentrations. In a Western blot analysis using the anti-Glycer-AGEs antibody after two-dimensional gel electrophoresis, various spots were detected in the control and fructose samples (Figure 1A and B). Western blot analysis using neutralized anti-Glycer-AGEs antibody was used to clarify the nonspecific spots, which showed no difference in their expression levels or expression pattern between the two samples (Figure

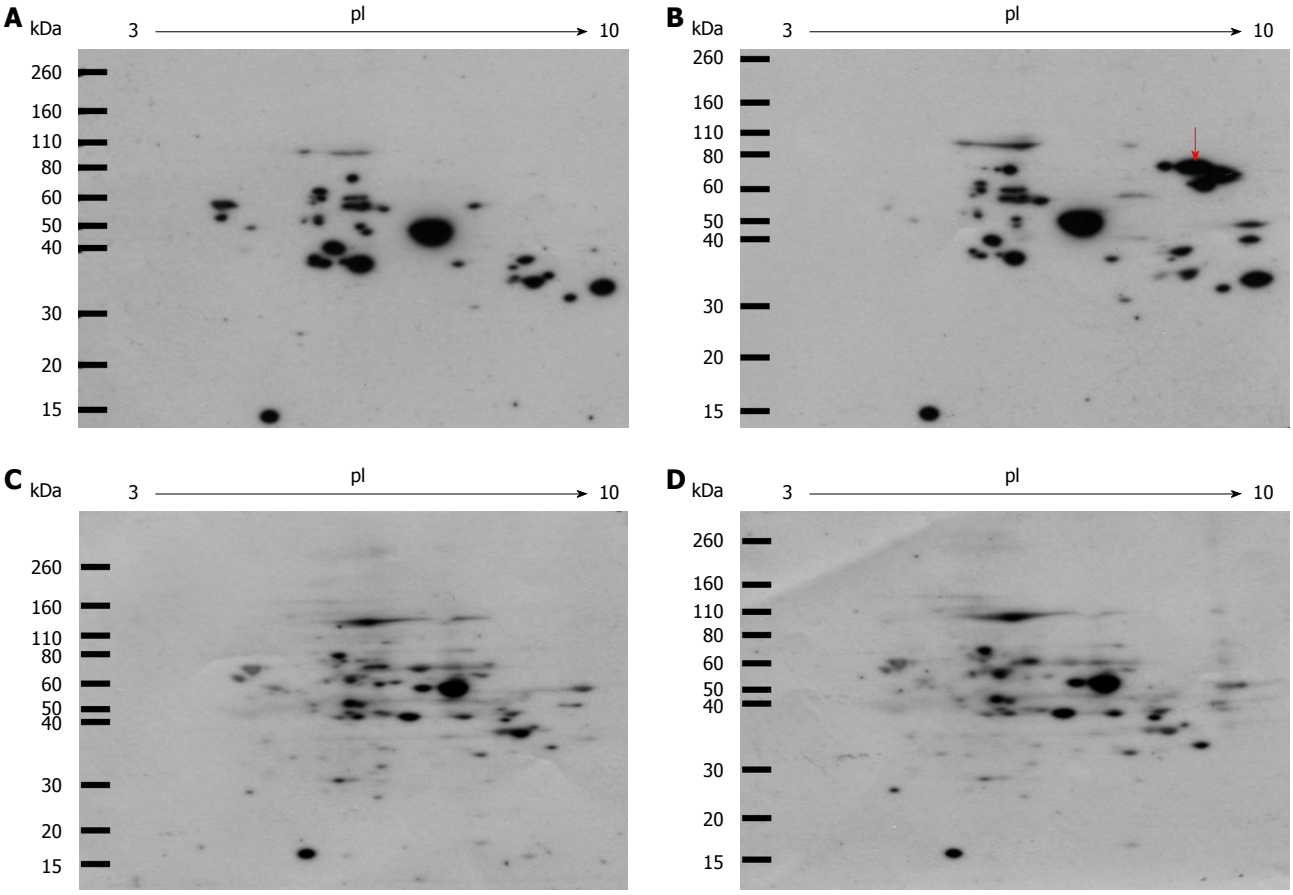

Figure 1 Western blot analysis of intracellular glyceraldehyde-derived advanced glycation end-products. Cells were incubated with or without 2 mmol/L fructose for 5 d. Cell lysates (100 μg of protein/gel) were separated on two-dimensional gradient sodium dodecyl sulfate-polyacrylamide electrophoresis and probed with Anti-glyceraldehyde-derived advanced glycation end-products (Glycer-AGEs) antibody (A, B), or anti-Glycer-AGEs antibody neutralized by an excess of Glycer-AGEs-bovine serum albumin (C, D); A,C: control samples; B,D: Fructose samples. The arrow shows spots where a difference was seen.

1C and D). In the sample that was exposed to high fructose concentrations, the Glycer-AGE spots were observed to have isoelectric points in the range of 8.0-9.0 and molecular weights in the range of 60-80 kDa (Figure 1B arrow).

Identification of the proteins modified by glyceraldehyde upon exposure to high fructose concentrations

After two-dimensional gel electrophoresis, for the sample exposed to high concentrations of fructose, the gel was stained with Coomassie blue (Figure 2). The gel spots that matched those detected with anti-Glycer-AGEs antibody were identified by mass spectrometry analysis. We identified four spots of Glycer-AGEs (Figure 2 circle), and interestingly, all four proteins were hnRNPM (Table 1). The spots detected with anti-Glycer-AGEs antibody were in accordance with the spots detected with anti-hnRNPM antibody (Figure 3).

hnRNPM is the target protein of glycation

We examined the effects of exposure to high fructose concentrations on the modification of hnRNPM by glyceraldehyde. Fructose exposure induced a concentration-dependent increase in glyceraldehyde modification of hnRNPM, but it did not increase the protein or mRNA levels of hnRNPM (Figure 4).

Next, we examined the effects of the modification of hnRNPM by glyceraldehyde, which is a precursor of Glycer-AGEs. After 5 d in culture, the cells were incubated with or without 4 mmol/L glyceraldehyde for 6 h. Western blot analysis using anti-Glycer-AGEs antibody after two-dimensional gel electrophoresis showed that the various spots of the glyceraldehyde sample were detected more strongly than the control sample (Figure 5A and B). The spot of hnRNPM was also included (Figure 5B arrow). Furthermore, Western blot analysis using anti-hnRNPM antibody showed the bands that shifted to the top, whereas the hnRNPM protein of 68 kDa was decreased by the addition of glyceraldehyde. On the other hand, such a change was not seen in β-actin or GAPDH (Figure 5C).

DISCUSSION

Glycer-AGEs cause various intracellular and extracellular adverse effects. Extracellular Glycer-AGEs interact with receptors for AGEs and increase oxidative stress by the production of reactive oxygen species in the cell[19]. On the other hand, intrace-

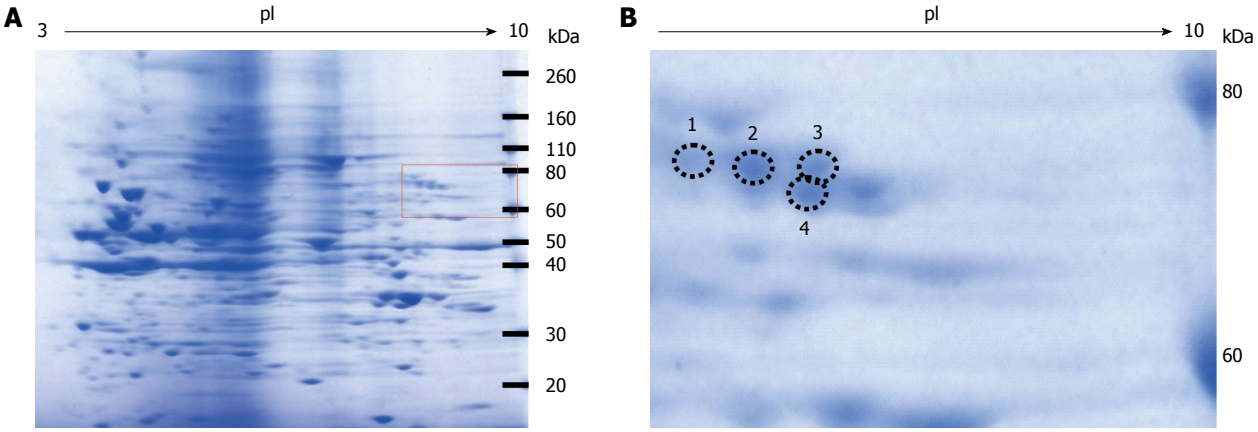

Figure 2 Coomassie blue staining of the two-dimensional gradient gel. A: High fructose exposure samples were separated on two-dimensional gradient sodium dodecyl sulfate-polyacrylamide electrophoresis and then stained with EzStain Aqua; B: Higher magnification of the square region of the left panel. The number and the position of the four selected spots are indicated by circles.

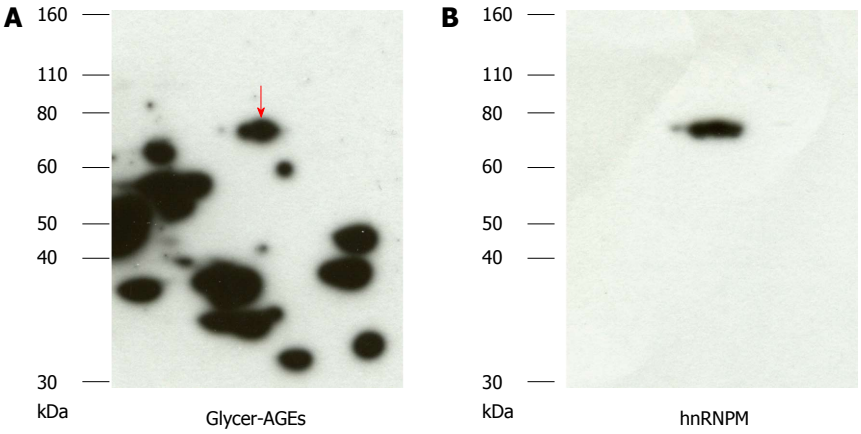

Figure 3 Detection of glyceraldehyde modification of heterogeneous nuclear ribonucleoprotein M by high fructose exposure. A: Samples exposed to high fructose concentrations were separated on two-dimensional gradient sodium dodecyl sulfate-polyacrylamide electrophoresis and probed with anti-glyceraldehyde-derived advanced glycation end-products (Glycer-AGEs) antibody. The arrow shows spots where a difference was seen; B: They were reprobed with heterogeneous nuclear ribonucleoprotein M (hnRNPM) antibody.

Table 1 Identification of intracellular glyceraldehyde-derived advanced glycation end-products formed by high fructose exposure

Spot No.	Protein name	Score	Mass values matched	Coverage	Calculated isoelectric point	Nominal mass (kDa)
1	Heterogeneous nuclear ribonucleoprotein M	90	13	20%	8.8	78
2	Heterogeneous nuclear ribonucleoprotein M	99	18	27%	8.8	78
3	Heterogeneous nuclear ribonucleoprotein M	84	16	28%	8.8	78
4	Heterogeneous nuclear ribonucleoprotein M	76	14	22%	8.8	78

Selected spots were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis. The number and the position of the four selected spots refer to the numbered spots in Figure 2B.

llular Glycer-AGEs cause a functional change in the protein itself[11,12]. We previously observed that glyceraldehyde, which is a precursor of Glycer-AGEs, increases the intracellular Glycer-AGEs in Hep3B cells. Among the intracellular Glycer-AGEs that were formed, heat shock cognate 70 was identified as a glyceraldehyde-modified protein, and the modification by glyceraldehyde reduced the activity of the protein[18].

In the liver, glyceraldehyde is believed to be produced by two pathways: the glycolytic pathway and the fructose metabolic pathway. Fructose metabolism involves fructokinase and is especially important in the liver after food intake. Fructose is phosphorylated to fructose-1-phosphate (F-1-P) by a specific kinase, and the liver aldolase B can cleave F-1-P to produce dihydroxyacetone phosphate and glyceraldehyde[20]. Therefore, the liver more readily accumulates glyceraldehyde than other organs. In this study, we have described the formation of intracellular Glycer-AGEs induced by high fructose exposure in Hep3B

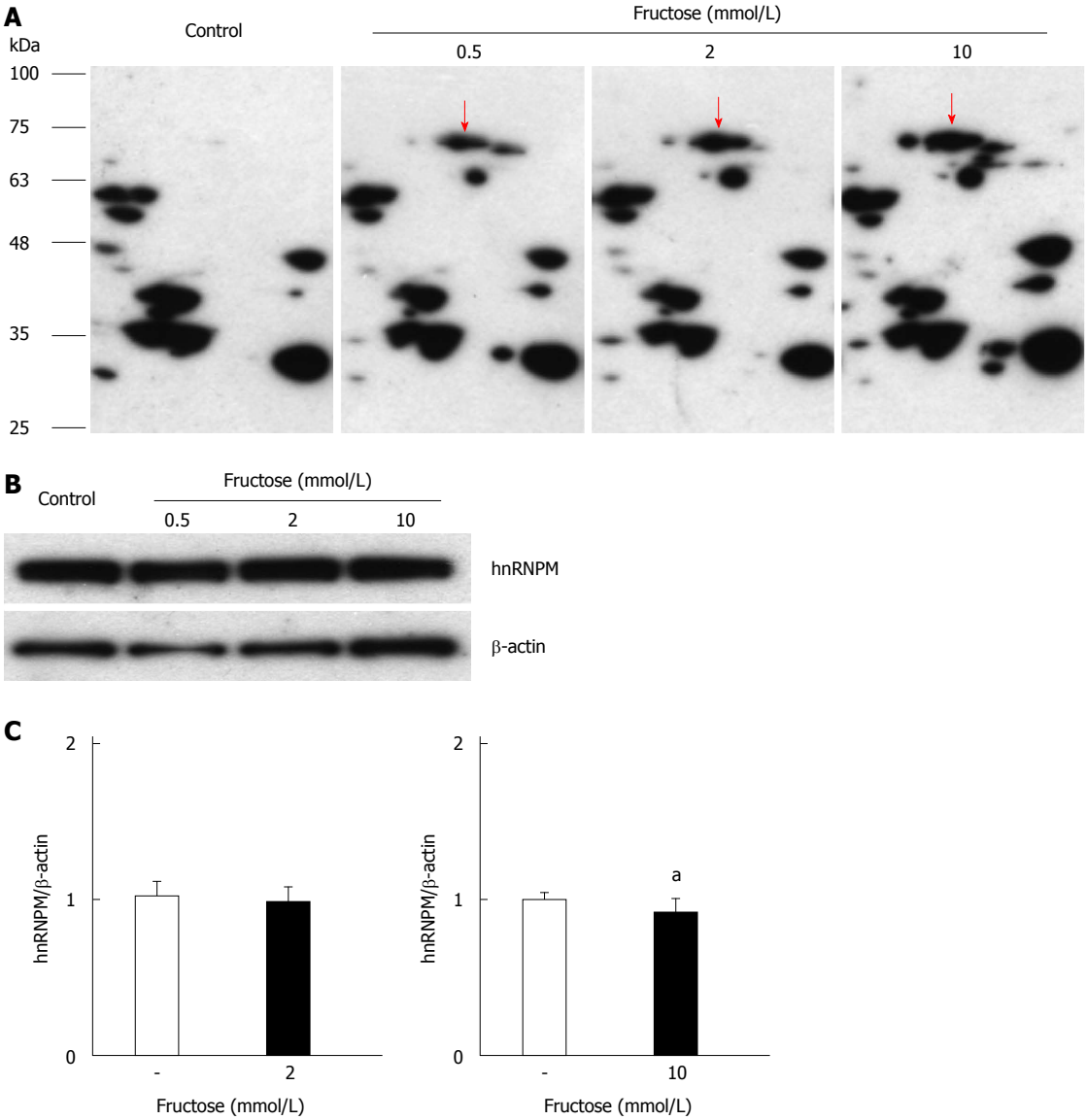

Figure 4 Effects of glyceraldehyde modification of heterogeneous nuclear ribonucleoprotein M by high fructose exposure. Cells were incubated with 0.5-10.0 mmol/L fructose for 5 d. A: Cell lysates were separated on two-dimensional gradient sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) and the glyceraldehyde modification of heterogeneous nuclear ribonucleoprotein M (hnRNPM) was determined by probing with anti-Glycer-AGEs antibody. The arrow shows spots identified with hnRNPM; B: Cell lysates were separated by SDS-PAGE and probed with anti-hnRNPM antibody. Equal protein loading was determined using anti-β-actin antibody; C: The levels of mRNA expression were analyzed by real-time RT-PCR, and the result was normalized to β-actin. Data are shown as mean ± SD (n = 6); $^{a}P < 0.05$ *vs* control. Left panel: With 2 mmol/L fructose, right panel: With 10 mmol/L fructose.

cells.

Whether the *in vitro* addition of fructose to hepatocytes is physiologically relevant is an important issue for further research. Le *et al*[21] demonstrated that the baseline fructose level in the human peripheral vein was about 5 μmol/L, and after ingestion of 24 oz of HFCS-sweetened beverages, this level increased to 0.3 mmol/L. Fructose absorbed in the small intestine is carried to the portal vein. Therefore, it is expected that the portal vein has higher fructose levels than the peripheral vein. Furthermore, Sugimoto *et al*[22] demonstrated that the peak concentration of portal fructose was approximately ten times that of peripheral fructose after sucrose ingestion in rats. This is consistent with our findings, which show that Glycer-AGEs were detected even when the concentration of fructose was less than 3 mmol/L.

Heterogeneous nuclear ribonucleoproteins, which directly bind to nascent RNA polymerase Ⅱ transcripts, play an important role in processing heterogeneous nuclear RNAs to form mature mRNAs and in regulating gene expression. It is known that hnRNPM appears as a cluster of four proteins, M1-4, of 64-68 kDa in two-dimensional gel electrophoresis[23]. We showed that a Glycer-AGE, which we identified to be hnRNPM, is a modified protein that was detected in cells exposed to high fructose concentrations, and this exposure induced a concentration-dependent increase in Glycer-AGEs (glyceraldehyde-modified

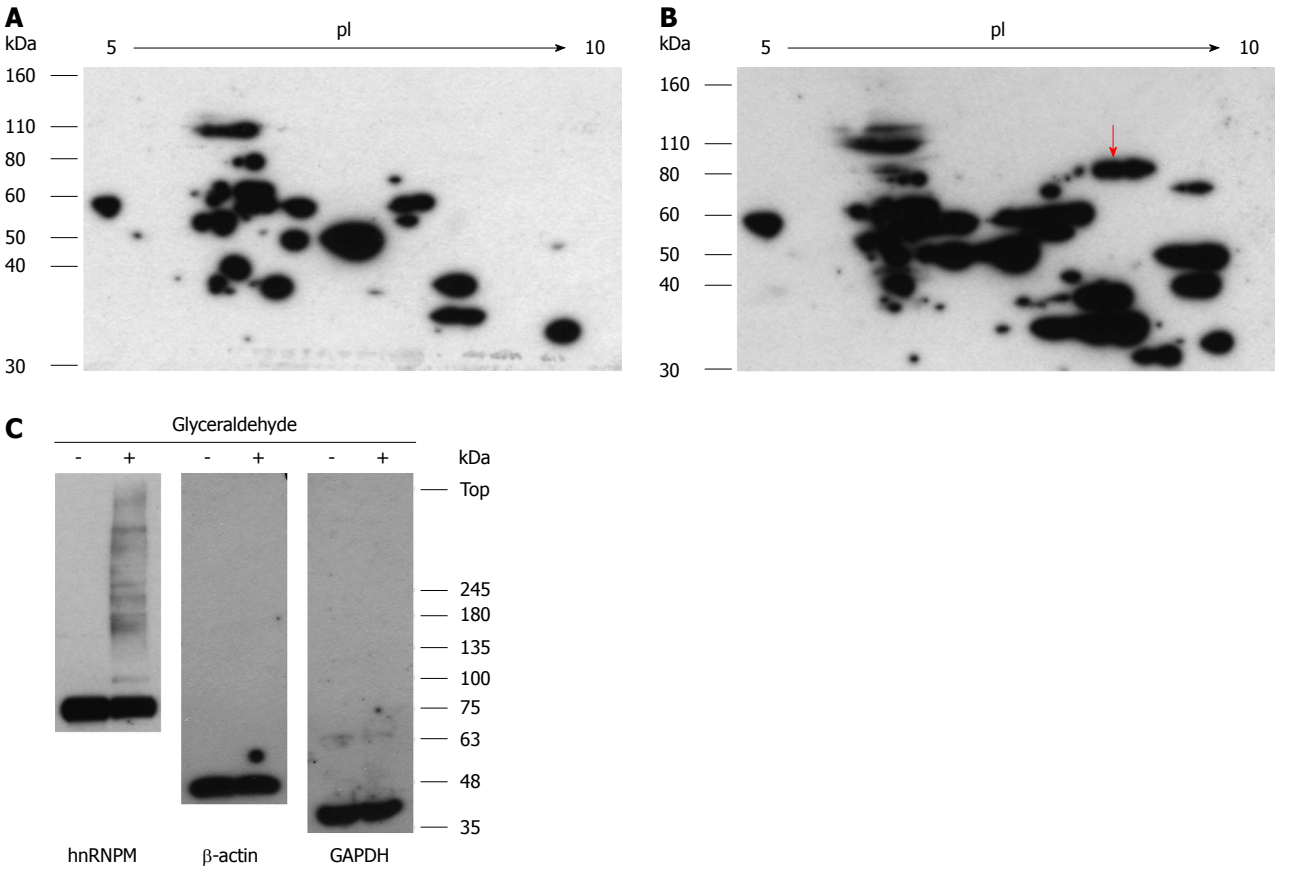

Figure 5 Effects of glyceraldehyde modification of heterogeneous nuclear ribonucleoprotein M. Hep3B cells were incubated for 5 d and incubated with or without 4 mmol/L glyceraldehyde for 6 h. Cell lysates were separated on two-dimensional gradient sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) (A, B); or SDS-PAGE and probed with anti-Glycer-AGEs antibody (C). A: Control sample; B: Glyceraldehyde sample. The arrow shows spot identified with heterogeneous nuclear ribonucleoprotein M (hnRNPM).

hnRNPM). It is known that the ketones or aldehydes of sugars can modify the side chains of lysine and arginine residues in proteins[24,25]; Cotham *et al*[26] demonstrated that dicarbonyl compounds react primarily with arginine residues. Lysine and arginine, amino acids that have a basic side chain, are important in RNA-protein interactions[27-31]. In particular, the function of hnRNPM may be to influence lysine sumoylation and arginine methylation, which are post-translational modifications[32,33]. Furthermore, hnRNPM possesses an unusual hexapeptide-repeat region rich in methionine and arginine residues (MR repeat motif)[23], and this motif may participate in the formation of Glycer-AGEs. In experiments where glyceraldehyde is externally added, the bands that shifted to the top appeared, while the level of 68-kDa hnRNPM protein was decreased. It is well known that the glycation of proteins forms intermolecular crosslinking[34-36]. Therefore, it is thought that this phenomenon of crosslinking characterizes glycation. However, no crosslinking was observed in β-actin or GAPDH. This suggests that the hnRNPM protein was more easily glycated than the other proteins.

Finally, in order to understand hnRNPM function, we used RIP-Chip analysis in Hep3B cells to identify the genes that are controlled by hnRNPM. The results showed that apolipoprotein E (APOE) and fibrinogen-like 1 (FGL1) were included in the top 50 such genes (Table 2). It is known that APOE is a ligand for low-density lipoprotein receptors and participates in the transport of cholesterol and other lipids[37]. It was reported that the APOE polymorphism is significantly associated with NASH[38]. Furthermore, APOE-deficient, APOE*3-Leiden (variant form of APOE), and APOE2 knock-in (APOE2ki) mice are widely used as models of liver steatosis[39-43]. These mice were maintained on a high-fat diet to allow the development of NASH[40-43]. It is also known that FGL1 expression is induced after liver injury; FGL1 stimulates hepatocyte proliferation and protects hepatocytes from injury[44-46].

In conclusion, among the intracellular Glycer-AGEs that were formed, hnRNPM was identified as a glyceraldehyde-modified protein, and it was more easily glycated than the other proteins. Therefore, we believe that the gene responsible for NAFLD and NASH is among those whose expression is regulated by hnRNPM. These results suggest that intracellular Glycer-AGEs may play a critical role in the pathogenesis of NAFLD and NASH and may serve as potential targets for therapeutic intervention. A

Table 2 Top 50 RNA targets of heterogeneous nuclear ribonucleoprotein M in Hep3B cells

Rank	Gene title	Gene symbol
1	Apolipoprotein E	*APOE*
2	Albumin	*ALB*
3	RNA, 28S ribosomal 1	*RN28S1*
4	PQ loop repeat containing 2	*PQLC2*
5	Zinc finger protein 865	*ZNF865*
6	Unknown	*A_33_P3396434*
7	lincRNA	*XLOC_l2_004940*
8	CD63 molecule	*CD63*
9	Eukaryotic translation elongation factor 1 alpha 1	*EEF1A1*
10	G protein-coupled receptor 155	*GPR155*
11	Family with sequence similarity 74, member A4	*FAM74A4*
12	LOC284600	*ENST00000448179*
13	LOC100506453	*ENST00000381105*
14	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit G	*ATP5L*
15	Unknown	*A_33_P3370515*
16	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 2	*PSMD2*
17	Ribosomal protein L23	*RPL23*
18	Proteasome (prosome, macropain) subunit, beta type, 1	*PSMB1*
19	Arsenic (+3 oxidation state) methyltransferase	*AS3MT*
20	Heat shock 60 kDa protein 1 (chaperonin)	*HSPD1*
21	H2A histone family, member V	*H2AFV*
22	lincRNA	*XLOC_l2_015885*
23	Fucosyltransferase 6 (alpha (1,3) fucosyltransferase)	*FUT6*
24	Cytochrome P450, family 2, subfamily W, polypeptide 1	*CYP2W1*
25	Unknown	*A_33_P3275826*
26	Microtubule-associated protein 4	*MAP4*
27	lincRNA	*XLOC_l2_002910*
28	X-ray repair complementing defective repair in Chinese hamster cells 6	*XRCC6*
29	Solute carrier family 16, member 12 (monocarboxylic acid transporter 12)	*SLC16A12*
30	Actin, beta	*ACTB*
31	Unknown	*A_33_P3315763*
32	Mitochondrial carrier 1	*MTCH1*
33	Stromal cell-derived factor 2-like 1	*SDF2L1*
34	Chromosome 19 open reading frame 10	*C19orf10*
35	Kinesin family member 1C	*KIF1C*
36	Fibrinogen-like 1	*FGL1*
37	RAN, member RAS oncogene family	*RAN*
38	Unknown	*ENST00000400768*
39	Homer homolog 3 (Drosophila)	*HOMER3*
40	HscB iron-sulfur cluster co-chaperone homolog (*E. coli*)	*HSCB*
41	Canopy 2 homolog (zebrafish)	*CNPY2*
42	Thymidine phosphorylase	*TYMP*
43	F11 receptor	*F11R*
44	Mastermind-like 1 (Drosophila)	*MAML1*
45	Electron-transfer-flavoprotein, beta polypeptide	*ETFB*
46	Heterogeneous nuclear ribonucleoprotein A1	*HNRNPA1*
47	Alpha-1-microglobulin/bikunin precursor	*AMBP*
48	Calreticulin	*CALR*
49	Solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3	*SLC25A3*
50	Alpha-2-HS-glycoprotein	*AHSG*

RNA-hnRNPM complexes were co-immunoprecipitated with anti-heterogeneous nuclear ribonucleoprotein M antibody bound to protein G-magnetic beads, and RNAs were isolated and identified by microarray analysis.

further analysis of the target genes of hnRNPM will be necessary in the future.

COMMENTS

Background

Excessive intake of fructose contributes to the development of nonalcoholic fatty liver disease and to the progression of the disease to nonalcoholic steatohepatitis (NASH). Fructose is metabolized to glyceraldehyde, which is a precursor of glyceraldehyde-derived (Glycer)-advanced glycation end-products (AGEs). AGEs are formed by the Maillard reaction, a nonenzymatic reaction that occurs between the ketones or aldehydes of sugars and the amino groups of proteins. AGEs are associated with aging and diabetes-related pathologic complications. The immunohistochemical analysis of Glycer-AGEs shows an intense staining in the livers of patients with NASH. However, the effects of fructose on the formation of intracellular Glycer-AGEs remain poorly understood.

Research frontiers

Evidence suggests that among the various AGEs, Glycer-AGEs are associated with diabetes-related pathologic complications, NASH, and cancer. The extracellular Glycer-AGEs-receptor for AGE signaling pathway is well understood, and it has been previously shown that AGE modifications adversely alter protein functions. In this study, the authors examined the formation of

intracellular Glycer-AGEs in the presence of high concentrations of fructose.

Innovations and breakthroughs

This study reported the formation of intracellular Glycer-AGEs and identified the glyceraldehyde-modified proteins by exposing the cells to high fructose concentrations.

Applications

The experimental data can be used in further studies for therapeutic intervention using these Glycer-AGEs as potential targets.

Peer-review

The study by Takino and coworkers focuses on the molecular mechanism with which excessive intake of fructose contributes to the development of an emerging and worrisome pathology such as nonalcoholic fatty liver disease and its inflammatory progression, NASH.

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ORIGINAL ARTICLE

Basic Study

Occult infection related hepatitis B surface antigen variants showing lowered secretion capacity

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Abstract

AIM: To elucidate the molecular mechanisms underlying hepatitis B virus (HBV) occult infection of genotype C.

METHODS: A total of 10 types of hepatitis B surface antigen (HBsAg) variants from a Korean occult cohort were used. After a complete HBV genome plasmid mutated such that it does not express HBsAg and plasmid encoding, each HBsAg variant was transiently co-transfected into HuH-7 cells. The secretion capacity and intracellular expression of the HBV virions and HBsAgs in their respective variants were analyzed using real-time quantitative polymerase chain reaction assays and commercial HBsAg enzyme-linked immunosorbent assays, respectively.

RESULTS: All variants exhibited lower levels of HBsAg secretion into the medium compared with the wild type. In particular, in eight of the ten variants, very low levels of HBsAg secretion that were similar to the negative control were detected. In contrast, most variants (9/10) exhibited normal virion secretion capacities comparable with, or even higher than, the wild type. This provided new insight into the intrinsic nature of occult HBV infection, which leads to HBsAg sero-negativeness but has horizontal infectivity. Furthermore, most variants generated higher reactive oxidative species production than the wild type. This finding provides potential links between occult HBV infection and liver disease progression.

CONCLUSION: The presently obtained data indicate that deficiency in the secretion capacity of HBsAg variants may have a pivotal function in the occult infections of HBV genotype C.

Key words: Occult infection; Hepatitis B virus; Hepatitis B surface antigen; Variants; Genotype C; Reactive oxidative species

Core tip: The presently obtained data indicate that deficiency in the secretion capacity of hepatitis B surface

antigen (HBsAg), but not virion, may have a pivotal function in occult infections of hepatitis B virus (HBV) genotype C, at least in occult infections in South Korea. This provided new insight into the intrinsic nature of HBV occult infections, which lead to HBsAg sero-negativeness but horizontal infectivity. In addition, reactive oxidative species production *via* possible induction of endoplasmic reticulum stress in hepatocytes provide a probable explanation for the links between occult infection and liver disease progression.

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INTRODUCTION

Hepatitis B virus (HBV) infection is a global health problem, and more than 350 million people are chronic carriers of the virus[1]. The infection is associated with a large spectrum of clinical manifestations ranging from acute or fulminant hepatitis to various forms of chronic infection, including asymptomatic carriers, chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC)[2,3]. South Korea is recognized as an endemic area of HBV infection[4]. Furthermore, extraordinary prevalence of genotype C2 has been reported in this area[5]; this genotype is known to be more virulent and more prone to mutation than genotype B[6], and it might contribute to the distribution of characteristic HBV mutation patterns related to the progression of liver diseases[7-16].

Occult HBV infection is defined as the infection state negative for hepatitis B surface antigen (HBsAg) serology, but it has shown viral genome persistence in infected individuals[17-19]. In general, HBV infection is diagnosed when the circulating HBsAg is serologically detected. However, recent progress in molecular-based technology, such as polymerase chain reaction (PCR)-based methods, has enabled HBV infection to be proven from HBsAg negative individuals with or without circulating antibodies to HBsAg and/or hepatitis B core antigen[20-22]. A large body of evidence has demonstrated that HBV occult infection is highly prevalent, particularly in HBV endemic areas, and it has distinct clinical entities. In particular, it is significantly related to severe forms of liver disease, such as cirrhosis and HCC[23,24]. Furthermore, in hepatitis C virus (HCV) infected patients, liver disease could worsen HCV infection[25-27].

Recently, there has been significant progress in obtaining an understanding of the molecular mechanisms that underlie occult HBV infection, together with increased clinical concerns throughout the world[26]. Among earlier findings, mutation in the HBsAg region, particularly in the "a" determinant, is regarded as a significant mechanism in relation to occult HBV infection. Structural alterations in HBsAg induced through mutations in or even outside the "a" determinant led to reduced affinity against the antibody in HBV diagnostic assays[26]. Furthermore, HBsAg mutations are known to contribute to HBV occult infections *via* reduction of virion secretion[27]. Because HBsAg variants are a serious health concern not only because they are not detectable by some commercial HBsAg assays, but also because they can infect vaccinated individuals, the emergence of HBsAg variants with novel mutations must be monitored among populations within HBV endemic areas, such as South Korea.

Recently, we reported various types of novel HBsAg variants of genotype C2 from South Korean occult subjects[14]. The present study elucidates the mechanisms related to the occult infection of ten types of genotype C2 HBsAg variants that were discovered in our previous study, primarily focusing on the extracellular secretion capacity of HBV virion and HBsAg.

MATERIALS AND METHODS

HBV DNA extraction and PCR amplification

For purification of the HBV DNA from serum, a QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) was used according to the manufacturer's instructions. Briefly, a 200 μL aliquot of obtained serum was incubated with QIAGEN protease and buffer AL at 65 ℃ for 10 min. The lysate was applied to a QIAamp spin column, spun, and washed three times with buffer AW, and finally eluted with 50 μL of BQW. In order to investigate the mutation patterns of the occult HBV infection, we conducted a nested PCR targeting the small surface open reading frame in Korean HBsAg-negative subjects. The first round of PCR was performed using the sense primer PreS2-Del-F2 (positions 2814-2832, 5′ - GGG TCA CCA TAT TCT TGG G - 3′) and the antisense primer HB2R (positions 970-989, 5′ - CAT ACT TTC CAA TCA ATA GG - 3′), which target a large surface region, while the second round of amplification was performed using the sense primer Cystein-S-F1 (positions 155-179, 5′ - ATG GAG AGC ACA ACA TCA GGA TTC C - 3′) and the antisense primer Cystein-S-R1 (positions 811-835, 5′ - TCA AAT GTA TAC CCA AAG ACA AAA G - 3′). The PCR was initiated using the hot-start technique in a 50 μL PCR mixture containing 2.5 mmol/L $MgCl_2$, 400 mmol/L dNTP, and 2.5 U of LA Taq polymerase (Takara Bio Inc., Shiga, Japan). The reaction mixture was subjected to 30 cycles of amplification (60 s at 95 ℃, 45 s at 52 ℃, and 90 s at 72 ℃) followed by a 5 min extension at 72 ℃. A 96-well thermocycler (Model 9600, Perkin-Elmer Cetus, Norwalk, United States) was also used. The obtained PCR products were analyzed *via* electrophoresis on 1% agarose gels, stained with

ethidium bromide, and visualized on a ultraviolet trans-illuminator.

Cloning and sequencing analysis of small surface ORFs

In order to analyze the mutation patterns of the small surface regions of 41 occult subjects, the 681-bp of the PCR products was cloned using a TOPO TA cloning kit (Invitrogen, Carlsbad, United States). The sequencing was performed using an Applied Biosystems model 377 DNA automatic sequencer (Perkin-Elmer Applied Biosystems, Warrington, United Kingdom). The mutations were classified through comparisons with seven reference strains obtained from GenBank [accession numbers (Genotypes) M57663 (A), AB100695 (B), AB074755 (C1), AY641558 (C2), X02496 (D), AB106564 (E), and X75663 (F)]. The nucleotides were aligned and their similarities were calculated using the multiple-alignment algorithm in Megalign (DNASTAR, Windows Version 3.12e).

Cloning of HBV small surface for expression

The small surface regions of ten subjects with novel mutation patterns related to the occult infection including the wild type (Nor) and pIRES2-EGFP only (mock) were cloned for the expression of HBsAg in the HuH-7 cell line. The TOPO TA cloned small surface region was re-cloned into the pIRES2-EGFP expression vector (Clontech, Mountain View, United States) including the cytomegalovirus (CMV) promoter as per the manufacturer's recommendation.

Cell culture and transient co-transfection

Human hepatoma cells (HuH-7) were cultured in Dulbecco's modified Eagle's medium (HyClone, Thermo Scientific, United States) containing 10% fetal bovine serum (GibcoBRL, Grand Island, United States) and 100 μg/mL of penicillin-streptomycin (GibcoBRL, Grand Island, United States). The cells were seeded on cell culture dishes (Falcon, San Jose, United States) and sub-cultured using a trypsin-EDTA (GibcoBRL, Grand Island, United States) treatment. All cells were maintained at 37 ℃ in a humidified 5% CO_2 atmosphere. In order to detect the HBsAg, viral DNA, and ROS level in the transient co-transfected HuH-7 using the novel mutants in this study, the cells (2×10^5) were seeded in 6-well plates. After incubation for 24 h, the cells were transiently co-transfected with pHY92-1.1x-HBV-deleted small surface plasmids (pHY92-delS) and pIRES2-EGFP-HBV small surface plasmids presenting occult HBV variants using Lipofectamine 2000 (Invitrogen, Carlsbad, United States) and incubated at 37 ℃ with 5% CO_2 for 48 h. After 48 h, the cell supernatant was collected and the pellets were washed with phosphate buffered saline (PBS) (GibcoBRL, Grand Island, United States). The washed pellets were lysed using mild Reporter Lysis Buffer (RLB) (Promega, WI, United States); these pellets were then used in the subsequent experiments.

Construction of HBV full genomic DNA with deleted HBsAg via site-directed mutagenesis

In order to verify the effects of the mutations related to the occult infections in HBV full genomic constructs, we created HBV full genome constructs that do not express HBsAg through site-directed mutagenesis of start codon of HBsAg (ATG to ATC). For this purpose, we used a plasmid and pHY92 vector containing a copy of the 1.1x-unit length HBV genome under the control of a CMV promoter (genotype A, serotype adw, HBV strain identical to GenBank AF305422), which was provided by Yang *et al*[28]. For each reaction, 5 ng of plasmid was incubated with 125 ng of mutagenic primer [the sense primer MUTA-Small-SF (position 134-175, 5′ - ACT GGG GAC CCT GCA CCG AAC ATC GAG AGC ACA ACA TCA GGA - 3′) and the anti-sense primer MUTA-Small-SR (same position, 5′ - TCC TGA TGT TGT GCT CTC GAT GTT CGG TGC AGG GTC CCC AGT - 3′)], 2.5 mmol/L of dNTP, and 2.5 U of Pyrococcus furiosus DNA polymerase (Takara Bio Inc., Shiga, Japan) with a final volume of 50 μL containing 10 mmol/L KCl, 6 mmol/L $(NH_4)_2SO_4$, 20 mmol/L Tris-HCl, 2 mmol/L $MgCl_2$, 1% Triton X-100, and 10 μg/mL bovine serum albumin. A cycling reaction was performed in a PCR machine (BIO-RAD, CA, United States). The reaction was initiated *via* heating for 5 min at 94 ℃. The reaction conditions for the denaturation, annealing, and elongation were 45 s at 94 ℃, 45 s at 52 ℃, and 10 min at 72 ℃, respectively, for a total of 15 cycles. The mixture was then subjected to digestion using 10 U of *Dpn*I restriction enzyme for 20 min at 37 ℃ and transformation into TOP10 (Invitrogen, Carlsbad, United States). Each bacterial colony was confirmed *via* DNA sequencing.

HBsAg ELISA

In order to compare the secretion capacity between the occult infection-related HBsAg variants, an ELISA was conducted for HBsAg, according to the given experiment method, using a commercial Bioelisa HBsAg color ELISA Kit (BIOKIT, Barcelona, Spain), MONOLISA HBs Ag ULTRA (BIO-RAD, CA, United States), and an ETI-MAK-4 HBsAg Enzyme Immunoassay Kit (DiaSorin, Saluggia, Italy) from the supernatant and lysed pellet. Furthermore, for normalization of the HBsAg ELISA in the cloned pIRES2-EGFP of the target HBsAg, we also measured the β-galactosidase expression level of the respective pIRES2-EGFP plasmid using a β-galactosidase enzyme assay system kit (Promega, WI, United States). β-galactosidase is a commonly used reporter molecule. The β-galactosidase enzyme assay system with RLB is a convenient method for assaying β-galactosidase activity in lysates prepared from cells transfected with β-galactosidase reporter vectors, such as the pSV-β-galactosidase control vector. For

normalization of pHY92-based full genomic constructs without the *EGFP* gene, the pSV-β-gal vector containing β-galactosidase was co-transfected; the β-galactosidase activities were analyzed according to the manufacturer's recommendation.

HBV viral DNA purification and Q-PCR analyses

The HBV DNA replication from the full genome HBV construct with pIRES2-EGFP clones was evaluated *via* quantitative real-time PCR targeting the secreted HBV viral DNA in the supernatant or intracellular viral DNA in a pellet of the cell culture. PCR amplification was performed with a set of real-time PCR primers targeting the small S gene designed to amplify a 101 base pair product and primer sequences as follows: the sense primer Real-SF (position 218-240, 5′ - TTG ACA AGA ATC CTC ACA ATA CC - 3′) and the antisense primer Real-SR (position 309-328, 5′ - GGA GGT TGG GGA CTG CGA AT - 3′). The culture medium was centrifuged in order to remove the cellular debris. Afterwards, the supernatant underwent ultracentrifugation at 20000 rpm in a SW28 rotor (Beckman Coulter, United States) for 2 h at 4 ℃. The collected pellet was re-suspended using PBS. The cell pellet was harvested using a trypsin-EDTA treatment and rinsed twice with PBS. The viral DNA was extracted using a Viral Gene-Spin DNA extraction Kit (iNtRON, Daejeon, South Korea) according to the manufacturer's instructions from the rinsed cell pellet and the collected viral particle of the supernatant. The quantitative PCR assay was conducted using commercial iQ SYBR Green supermix (Bio-Rad, CA, United States) and primers specific to the S gene. The HBV DNA levels were analyzed with an Exicycler™ 96 Real Time Quantitative Thermal Block system (Bioneer, Daejeon, South Korea). Each PCR was conducted in duplicate in a 25 μL volume using the iQ SYBR Green supermix for 5 min at 95 ℃ for the initial denaturing, followed by 40 cycles of 95 ℃ for 15 s and 56 ℃ for 15 s. Detection of the fluorescence was set at the last step of each cycle. In order to determine the specificity of the amplification, a melting curve analysis was applied to all final PCR products after the cycling protocol. The results are representative of three independent experiments.

ROS measurement

Dihydrorhodamine123 (DHR123; Calbiochem, San Diego, United States) is a cell-permeable fluorogenic probe that is useful for detecting ROS such as peroxide and peroxynitrite. It is not fluorescent until oxidized by ROS to the highly fluorescent product Rhodamine123. The formation of Rhodamine123 can be monitored using a fluorescence spectroscopy with excitation and emission wavelengths of 500 and 536 nm, respectively. For the ROS level detection in the transient transfected HuH-7 using the novel mutants from this study, the cells (2×10^5) were seeded in 6-well plates. The day after the transfection, the cells were treated with DHR123 at a final concentration of 10 μmol/L for 30 min and lysed with a ROS lysis buffer (5 mmol/L KH_2PO_4, 0.1 mmol/L EDTA, 0.1% Triton-X-100). Then, 100 μL of the cell lysate was transferred to Nunc immuno-microwell 96-well polystyrene plates (Nunclon, Carlsbad, United States) and analyzed using TECAN Infinite m200 pro (TECAN, Seestrasse, Switzerland) at an excitation of 500 nm and emission of 536 nm.

Ethics statement

This retrospective study was reviewed and approved by the Institutional Review Board of Seoul National University Hospital (IRB Grant No. C-0803-013-237), and the patients' medical records were anonymized and de-identified prior to the analyses.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism v5.01 (GraphPad software, SD, United States) and figures were generated using the same program. Tables were prepared using Excel 2010 (Microsoft, United States). For continuous variables, a one-way ANOVA was used when the data exhibited a normal distribution. The results are expressed as a comparative ratio, the mean ± SD, or as medians (range). A P value of < 0.05 (two-tailed) was considered to be statistically significant.

RESULTS

Mutation patterns of 10 HBsAg variants from Korean occult subjects

Previously, from 41 of 624 occult subjects (6.6%), we obtained the PCR amplicons using a nested PCR strategy targeting the large surface proteins (LHBs)[14]. Finally, we selected ten unique types of HBsAg variant of genotype C *via* sequence analysis of amplified LHBs, which have not been reported in other studies, and these were used to elucidate the occult HBV infection mechanism. The respective variants were denoted by abbreviations derived from the distinct mutations in this study. The mutation patterns of the ten HBsAg variants and patient information were determined through comparisons with the amino acids of a reference strain from genotypes A to F in the same region. Briefly, in five (KD, LL, 172R, 182L, and STOP) of the ten HBsAg variants, the mutations were only located outside the "a" determinant (aa 124 to 147). It is noteworthy that two variants (PAHS and STOP) had a premature mutation of the 182th codon (sW182*), which has been reported to be related to the clinical severity of genotype C infected chronic patients[12]. The PAHS and STOP variants had a total of eight and two mutations including sW182* in the HBsAg, respectively. In addition, 182L had a substitution in the 182th codon (W→L), which has only been reported to exist in occult cases in South

Table 1 Mutation patterns of ten variants from Korean occult subjects found in the hepatitis B surface antigen region

Number	17	32	33	36	44	47	52	56	61	66	73	85	101	107	111	117	126	127	129	130	134	140	145	146	158	170	172	177	182	195	204	206	220	227
Aminoacid	A	L	D	W	G	T	N	P	S	P	R	F	Q	C	P	S	I	P	Q	G	F	T	G	N	F	F	W	V	W	I	S	Y	F	★
Genotype A	·	·	·	·	·	V	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	★
Genotype B	·	·	·	·	E	V	·	Q	·	·	·	C	·	·	·	·	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	★
Genotype C1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	★
Genotype C2	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	★
Genotype D	·	·	·	·	·	V	·	·	·	·	·	·	·	·	·	·	T	·	·	·	Y	·	·	·	·	·	·	·	·	·	·	·	·	★
Genotype E	·	·	·	·	·	V	·	·	·	·	·	·	·	·	·	·	T	L	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	★
Genotype F	·	·	·	·	·	R	·	·	·	·	·	·	·	·	·	·	A	L	·	·	·	·	·	·	·	·	·	·	·	·	N	·	·	★
Nor (WT)	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	★
TN	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	T	·	N	·	·	·	·	·	·	·	·	·	·	·	·	·	·	★
TALR	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	A	·	·	L	·	·	·	·	·	·	R	·	★
ALK	·	·	G	·	·	A	·	·	L	·	·	·	K	·	·	·	·	·	·	·	·	·	·	D	·	·	·	·	·	M	·	·	·	★
KD	·	·	·	L	·	K	D	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	★
CNR	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	N	·	·	·	R	·	·	·	·	·	·	·	·	·	·	·	·	·	★
LL	·	·	·	·	·	·	·	·	·	·	·	·	·	·	L	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	L	★
PAHS	·	P	·	·	·	A	·	·	·	H	·	S	·	W	·	·	·	·	·	·	·	·	R	·	·	P	·	·	★					
172R	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	R	·	·	·	·	·	·	★
182L	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	L	·	·	·	·	★
Stop	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	A	★					

Black asterisk (★): Stop codon; Nor (WT): Normal (wild-type); TN: I126T, Q129N; TALR: A17T, T140A, F158L, Y206R; ALK: D33G, T47A, S61L, Q101K, N146D, I195M; KD: W36L, T47K, N52D; CNR: R73C, S117N, G130R; LL: P111L F220L; PAHS: L32P, T47A, P66H, F85S, C107W, G145R, F170P; 172R: W172R; 182L: W182L; Stop: V177A, W182Stop.

Korea, but not in chronic patients (Tables 1 and 2)[14].

Comparison of HBsAg and virion secretion capacity according to occult infection-related HBsAg variants

In order to address the issue of whether the occult HBV infection may be attributed to a reduced secretion capacity of the occult infection-related HBsAg variants, we analyzed the HBsAg secretion capacity of ten different types of HBsAg variants using a commercial HBsAg ELISA kit in a transient co-transfection system with both the pHY92-1.1x-full HBV genome construct (Genotype A, GeneBank No.AF305422), which is not capable of HBsAg expression *via* abrogation of the start codon (pHY92-delS), and the pIRES2-EGFP-HBV small surface plasmid vector into the HuH-7 cell (Figure 1).

All ten HBsAg variants exhibited a significantly reduced secretion capacity of HBsAg into the media compared with the wild type. In particular, eight variants, excluding the two variants TN and TALR, could not be secreted into the media, which is similar to the secretion level seen in the negative control (MOCK). This indicates that the HBV occult infections from these subjects may result from the lack of HBsAg secretion capacity. The eight variants exhibiting an HBsAg secretion capacity similar to the negative level were divided into two groups according to the detected intracellular HBsAg level. One group included two variants (ALK and KD) that exhibited higher levels of intracellular HBsAg than the wild type, which indicated that their reduced HBsAg secretion might result from increased intracellular accumulation of the HBsAg variants. The second group included six variants (CNR, LL, PAHS, 172R, 182L, and STOP) that exhibited very low levels of intracellular HBsAg, similar to the negative control (MOCK), and this indicated that their reduced secretion level might result from a problem of HBsAg protein stability or changed antigenicity through the mutations. Meanwhile, in order to investigate the effect of the occult-related variants on virion formation, we verified the amounts of viral DNAs from the medium and cell lysates of co-transfected HuH-7 cells using a real-time quantitative-DNA PCR. The results revealed that the level of extracellular viral DNA from nine occult-related variants (except "STOP") was almost equal to (ALK, KD, 172R, and 182L) or higher (TN, TALR, CNR, LL, and PAHS) than that of the wild type.

Table 2 Clinical, genotype, hepatitis B virus bDNA, and serologic data of the ten subjects with hepatitis B surface antigen variants used in the present study

Samples	Age	Sex	Genotype	HBV bDNA (copies/mL)	HBsAg	Anti-HBs	Mutations	Abbreviation
1	60	F	C2	< 2000	Negative	Negative	I126T, Q129N	TN
2	53	M	C2	< 2000	Negative	Negative	A17T, T140A, F158L, Y206R	TALR
3	60	F	C2	< 2000	Negative	Positive	D33G, T47A, S61L, Q101K, N146D, I195M	ALK
4	54	M	C2	< 2000	Negative	Negative	W36L, T47K, N52D	KD
5	44	M	C2	< 2000	Negative	Positive	R73C, S117N, G130R	CNR
6	41	F	C2	< 2000	Negative	Negative	P111L, F220L	LL
7	42	M	C2	< 2000	Negative	Negative	L32P, T47A, P66H, F85S, C107W, G145R, F170P, W182Stop	PAHS
8	73	M	C2	< 2000	Negative	Negative	W172R	172R
9	30	M	C2	< 2000	Negative	Positive	W182L	182L
10	3	F	C2	< 2000	Negative	Negative	V177A, W182Stop	Stop

HBsAg: Hepatitis B surface antigen; Anti-HBs: Anti-hepatitis B surface; F: Female; M: Male; HBV: Hepatitis B virus.

Table 3 Classification of the ten novel mutants according to the secretion level of hepatitis B surface antigen and viral DNA formation capacity

Grouping	Mutants	sAg level		Viral DNA level	
		Extracellular	Intracellular	Extracellular	Intracellular
Ⅰ	TN	Positive	Positive	Higher than WT	Similar to WT
	TALR	Positive	Positive	Higher than WT	Similar to WT
Ⅱ	ALK	Negative	Highly positive	Similar to WT	Similar to WT
	KD	Negative	Highly positive	Similar to WT	Similar to WT
Ⅲ	CNR	Negative	Weakly positive	Higher than WT	Similar to WT
	LL	Weakly Positive	Weakly positive	Higher than WT	Higher than WT
	PAHS	Negative	Negative	Higher than WT	Similar to WT
Ⅳ	172R	Negative	Weakly positive	Lower than WT	Lower than WT
	182L	Negative	Weakly positive	Lower than WT	Lower than WT
Ⅴ	Stop	Negative	Negative	Negative	Negative

sAg: Surface antigen; WT: Wild type.

This indicates that most occult-related HBsAg variants do not affect the HBV virion formation. The levels of intracellular viral DNA of seven variants (TN, TALR, ALK, KD, CCNR, LL, and PAHS) were equal to or higher than that of the wild type. However, the remaining three variants (172R, 182L, and STOP) exhibited very low levels of intracellular and extracellular HBsAg, and they also exhibited significantly lower intracellular viral DNA levels than that of the wild type (Figure 2).

Collectively considering the effects of the occult infection-related HBsAg variants on HBsAg and HBV virion formation, the ten different types of HBV variants analyzed in this study were categorized into five groups. Group Ⅰ included two variants (TN and TALR) and they displayed intracellular and extracellular HBsAg secretion and viral DNA levels similar to that of the wild type. Group Ⅱ also included two variants (ALK and KD) that exhibited very low levels of HBsAg secretion, but a higher level of accumulated intracellular HBsAg compared with the wild type. Group Ⅲ included three variants (CNR, LL, and PAHS) that exhibited very low levels of both secreted and intracellular HBsAg secretion, but similar levels of viral DNA compared with the wild type. Group Ⅳ included two variants (172R and 182L) that exhibited very low levels of both secreted and intracellular HBsAg secretion, and also lower levels of viral DNA compared with the wild type. Group Ⅴ included only the STOP variant, and it cannot produce HBsAg or virions in intracellular or secreted forms (Table 3).

Comparison with ROS production between the ten variants and the wild type

Alteration of the secretion capacity of the HBsAgs and HBV virions could lead to ROS production *via* the endoplasmic reticulum (ER) stress pathway[29,30]. In order to address the issue of whether the ten HBsAg variants used in this study could lead to ROS production compared with the wild type, we compared the ROS production of the 11 types of HBsAg (the ten variants and the wild type) using a DHR123 system, 24 h after co-transfection of both plasmids of pHY92-delS and pIRES2-EGFP-HBV small surface plasmid presenting occult HBV variants, respectively. Although generally all variants produced higher levels of ROS compared with the wild type, the ROS production differed substantially among the variants. While seven variants (TN, TALR, ALK, KD, CCNR, LL, and PAHS) belonging to Groups Ⅰ, Ⅱ, and Ⅲ produced relatively high levels of ROS production, the remaining three variants (172R, 182L, and STOP) belonging to Groups Ⅳ and Ⅴ produced ROS levels similar to that of the

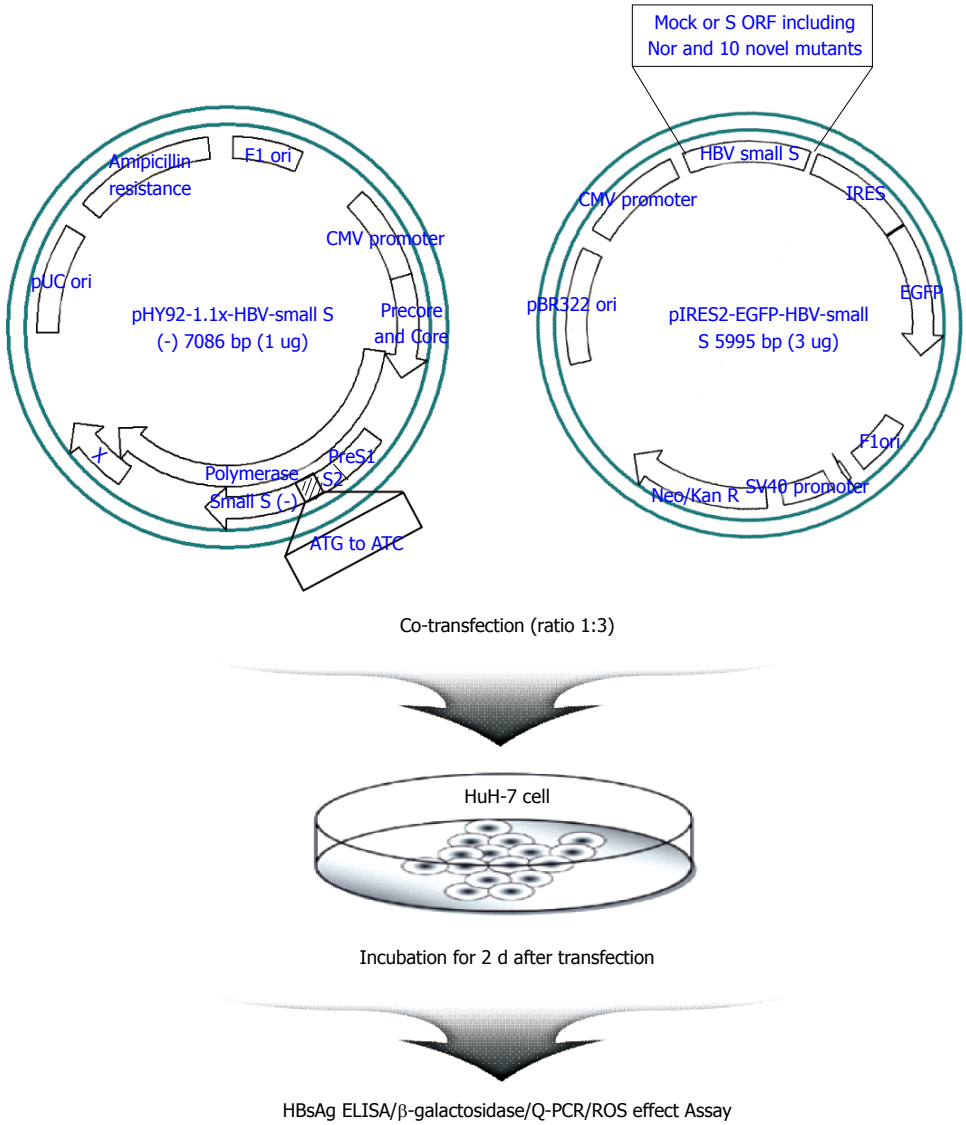

Figure 1 Schematic experimental strategy used in this study. Briefly, one microgram of pHY92-1.1x-HBV-small S (-) having a full-genome of hepatitis B virus (HBV) with a knock-out small surface open reading frame (ORF) was co-transfected with three micrograms of pIRES2-EGFP-HBV-small S expressing the sub-genome of small surface region, which has ten variants including mock and wild-type into HuH-7 cell line transiently. After co-transfection, HuH-7 cells were incubated for 2 d. Supernatant and lysed pellet were collected and used for various assays. The tests were performed in triplicate. HBsAg: Hepatitis B surface antigen; ELISA: Enzyme linked immunosorbent assay; Q-PCR: Quantitative polymerase chain reaction; ROS: Reactive oxygen species; Neo/Kan R: Neomycin and Kanamycin resistance.

wild type. In particular, the ROS production of two variants (KD and PAHS) was statistically significantly higher than that of the wild type (Figure 3).

DISCUSSION

To date, studies of occult infection related HBsAg mutations have primarily focused on mutations in the "a" determinant to produce the occult infection or generation of vaccine escape variants through reduction of the binding affinity between the HBsAg and antibody to HBsAg[31-34]. Among these, the mutation from glycine to arginine in the 145th codon of HBsAg (*i.e.*, G145R) has been most frequently encountered[33,34]. However, this is not the case in South Korea where the genotype C infection is prevalent. It has been reported that the mutation of G145R is not responsible (but if so, rarely) for the occult infection in Korean subjects. Our previous report demonstrated that, in South Korea, the HBsAg variants of genotype C related to the occult infection harbored multiple mutations within HBsAg, primarily located outside the "a" determinant[14]. In this study, we attempted to uncover the molecular mechanism underlying the occult infection of HBV endemic areas such as South Korea using ten novel types of HBsAg variants from Korean subjects.

The transient transfection study using a total of ten novel HBsAg variants offers several noteworthy findings. First, defects in the HBsAg secretions may be a significant mechanism underlying occult infections. Although all variants exhibited lower levels of HBsAg secretion capacity, substantial differences between types of HBsAg variants were found. The

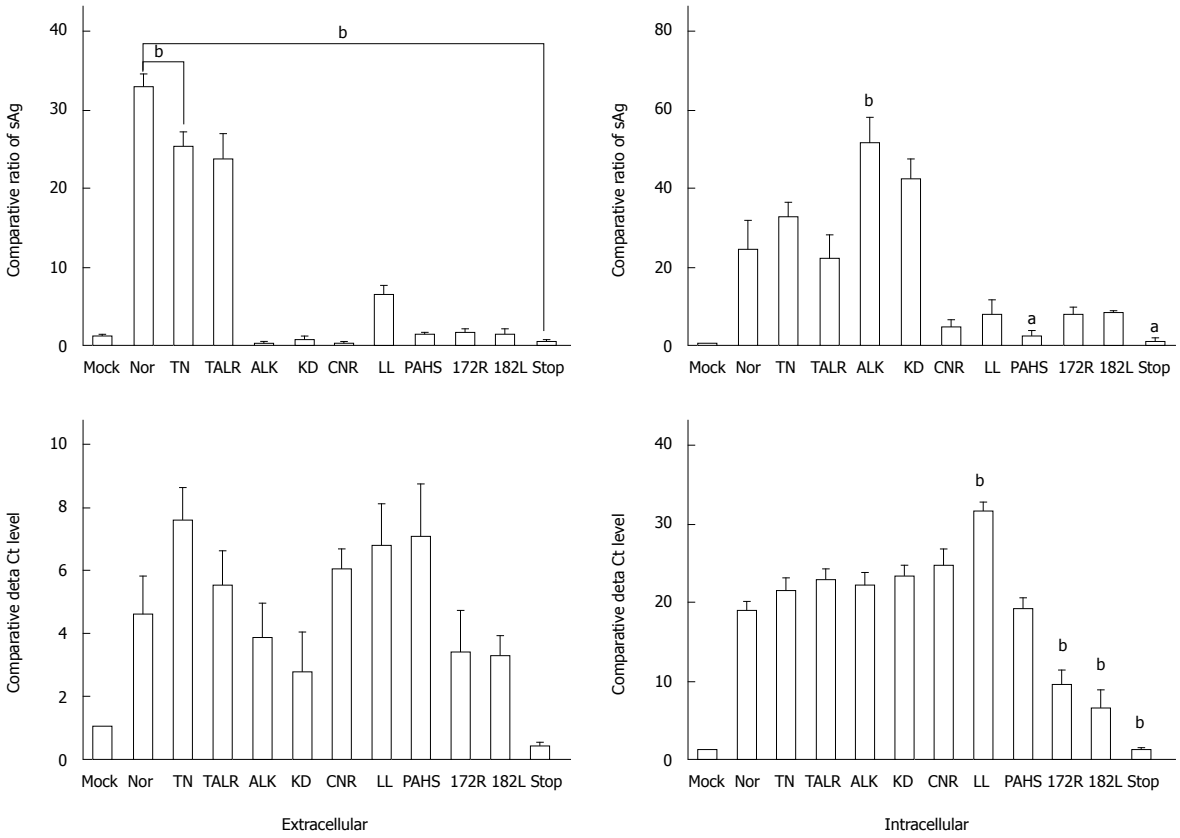

Figure 2 Secretion capacity and viral DNA formation of occult hepatitis B surface antigen variants. Extracellular secreted hepatitis B surface antigen (HBsAg) and intracellular expressed HBsAg from cell lysate were measured using a commercial HBsAg enzyme linked immunosorbent assay kit normalized *via* a β-galactosidase assay. After purification of the viral DNA from the supernatant and cell lysate using a total viral DNA preparation kit, detection of the viral DNA from both intracellular and extracellular was performed using real-time quantitative DNA-polymerase chain reaction assays. The hepatitis B virus DNA was normalized *via* a β-galactosidase assay. The tests were performed in triplicate (mean ± SD). sAg: Surface antigen. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* wild type (Nor) group.

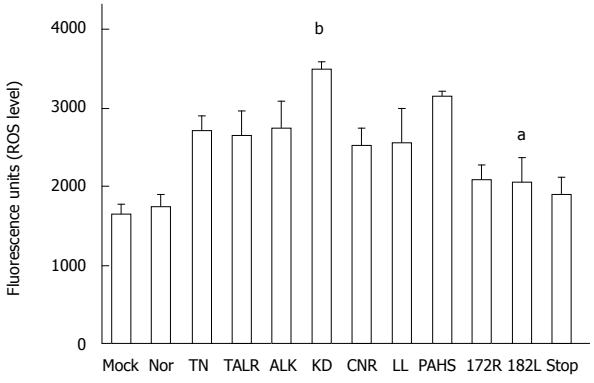

Figure 3 Effect of occult hepatitis B surface antigen variants on the reactive oxygen species system. The reactive oxygen species level according to the occult hepatitis B virus variants in transient transfected HuH-7 was measured using 20 μmol/L of DHR123 reagent, which can detect reactive oxygen species (ROS) such as peroxide and peroxynitrite. The tests were performed in triplicate (mean ± SD). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* wild type (Nor) group.

two variants of Group Ⅱ (KD and ALK) exhibited high levels of intracellular accumulation, but not secretion, which could lead to ER accumulation of the mutated HBsAg without protein degradation *via* an impaired ER-associated degradation process[29]. The six variants of Groups Ⅲ, Ⅳ, and Ⅴ exhibited low levels of HBsAg in both intracellular and secretory forms. For this, mechanisms such as antigenicity modification or protein stability defects could be proposed. Considering most variants (four of the six) did not have "a" determinant mutations, defects in protein stability of the variants rather than antigenicity modification is more probable. Furthermore, despite the defects of the HBsAg secretion capacity, most variants had potential for virion secretion comparable with or even higher than that of the wild type. This indicates higher normal infectivity, but was not detectable using the commercial HBsAg ELISA Kit. However, it could provide a probable explanation regarding the relatively high levels of occult infection prevalence in South Korea, as reported previously[14].

Second, our previous report demonstrated that, including the W182L and sW182* mutations, the 182nd codon was the most frequently affected among the mutations of HBsAg in the occult subjects (15/41 subjects). sW182* was also reported to be related to liver disease progression in Korean chronic patients[12]. However, W182L was distinct in the occult infection, because it was not found in chronic Korean patients[14]. Three types of variants with mutations

in the 182nd codon (PAHS and 182L of Group Ⅳ, and STOP of Group Ⅴ) were included in this study. There were substantial differences in the virion formation between PAHS with eight mutations and STOP with two mutations, although both had sW182*. In STOP, HBsAg production and virion were not found, as demonstrated in the previous report using the full HBV pHY92 construct with sW182* generated using site-directed mutagenesis[12], which indicates abortive production of mutated HBsAg. However, PAHS exhibited recovered HBV virion formation despite having sW182* even with a higher secretory virion level than the wild type, which indicates a role of additional mutations for virion formation. Despite the single mutation, 182L also exhibited a secretion profile similar to that of PAHS, with secretory HBV virion, but without HBsAg secretion. Therefore, collectively, our data proved that the 182nd codon has a pivotal function in HBsAg secretion. Furthermore, our data also provided a probable explanation regarding why mutation in the 182th codon is so prevalent in the occult subjects.

Third, considering previous reports that implicated reduced HBsAg in the induction of the ER stress pathway[29], HBsAg secretion deficiency *via* accumulation of occult-related HBsAg mutations may also provide links between the occult infection and progression of liver disease. In order to address this issue, we compared the ROS production that was expected to be induced by the ER stress pathway between the ten HBsAg variants and the wild type. Our data strongly supported the hypothesis that occult-related HBsAg variants could induce ROS induction in hepatocytes *via* the ER stress pathway, which increases the potential of liver disease progression; however, our data also demonstrated disparities in potentials to elicit ROS production in the hepatocytes among the HBV variants. This indicates differences in the ER stress inducing potentials and kinetics according to the mutation patterns of the HBsAg variants. The exact role of the respective mutation in inducing the ER stress-ROS axis remains to be elucidated in a future study.

In conclusion, our data indicate that deficiency in the secretion capacity of HBsAg, but not virion, that is induced by HBsAg mutations (particularly outside the "a" determinant) may have a pivotal function in occult infections of HBV genotype C, at least in occult infections in South Korea. Furthermore, it also provided new insight into the intrinsic nature of HBV occult infections, which leads to HBsAg seronegativeness but horizontal infectivity. In addition, proving ROS production *via* possible induction of ER stress by HBsAg variants in hepatocytes would provide a probable explanation for the links between occult infection and liver disease progression.

COMMENTS

Background

A large body of evidence has demonstrated that occult hepatitis B virus (HBV) infection is highly prevalent, particularly in HBV endemic areas. Recently, various types of novel hepatitis B surface antigen (HBsAg) variants of genotype C2 from Korean occult subjects have been introduced. This study elucidates the mechanisms related to occult infection of genotype C2 HBsAg variants introduced in a previous study, primarily focusing on the extracellular secretion capacity of HBV virion and HBsAg.

Research frontiers

These data indicate that deficiency in the secretion capacity of HBsAg, but not virion, that is induced by the HBsAg mutations (particularly outside the "a" determinant) may have a pivotal function in occult infections of HBV genotype C, at least in occult infections in South Korea.

Innovations and breakthroughs

Recent reports have highlighted the importance of "a" determinant mutations such as G145R as a major mechanism underlying occult infection. However, it is not the case in Korean occult subjects. This is the first study to report that deficiency in the secretion capacity of HBsAg variants (particularly outside the "a" determinant) may have a pivotal function in occult infections of HBV genotype C. Furthermore, this study proved reactive oxygen species production *via* possible induction of endoplasmic reticulum stress by HBsAg variants in hepatocytes, providing a probable explanation for the links between occult infection and liver disease progression.

Applications

The co-transient transfection system of both HBV full genomic DNA and occult infection related HBsAg variants introduced in this study could be effectively used for the development of a novel HBsAg detection method or for the screening of new vaccine escape or occult infection related mutants.

Terminology

Occult HBV infection is defined as the infection state negative for HBsAg serology, but it has shown viral genome persistence in infected individuals. In general, HBV infection is diagnosed when the circulating HBsAg is serologically detected. However, recent progress in molecular-based technology has enabled HBV infection to be proven from HBsAg negative individuals with or without circulating antibodies to HBsAg and/or hepatitis B core antigen.

Peer-review

The authors examined the underlying mechanism of HBV occult infection focusing on ten HBsAg variants from Korean occult subjects, recently introduced by the authors. It was revealed that all variants exhibited lower levels of HBsAg secretion into the medium, but similar level of virions, compared with the wild type. Furthermore, most variants generated higher reactive oxidative species production than the wild type. The results are interesting and may represent a molecular mechanism of HBV genotype C occult infection.

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ORIGINAL ARTICLE

Basic Study

Overexpression of B7-H3 augments anti-apoptosis of colorectal cancer cells by Jak2-STAT3

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Abstract

AIM: To investigate the role of the overexpression of *B7-H3* in apoptosis in colorectal cancer cell lines and the underlying molecular mechanisms.

METHODS: SW620 cells that highly overexpressed B7-H3 (SW620-B7-H3-EGFP) and HCT8 cells stably transfected with B7-H3 shRNA (HCT8-shB7-H3) were previously constructed in our laboratory. Cells transfected with pIRES2-EGFP were used as negative controls (SW620-NC and HCT8-NC). Real-time PCR and western blotting analysis were used to detect the mRNA and protein expressions of the apoptosis regulator proteins Bcl-2, Bcl-xl and Bax. A cell proliferation assay was used to evaluate the survival rate and drug sensitivity of the cells. The effect of drug resistance was detected by a cell cycle assay. Active caspase-3 western blotting was used to reflect the anti-apoptotic ability of cells. Western blotting was also performed to determine the expression of proteins associated with the Jak2-STAT3 signaling pathway and the apoptosis regulator proteins after the treatment with AG490, a Jak2 specific inhibitor, in B7-H3 overexpressing cells. The data were analyzed by GraphPad Prism 6 using a non-paired *t*-test.

RESULTS: Whether by overexpression in SW620 cells or downregulation in HCT8, B7-H3 significantly affected the expression of anti- and pro-apoptotic proteins, at both the transcriptional and translational levels, compared with the negative control ($P < 0.05$). A cell proliferation assay revealed that B7-H3 overexpression increased the drug resistance of cells and resulted in a higher survival rate ($P < 0.05$). In addition, the results of cell cycle and active caspase-3 western blotting proved that B7-H3 overexpression inhibited apoptosis in colorectal cancer cell lines ($P < 0.05$). B7-H3 overexpression improved Jak2 and STAT3 phosphorylation and, in turn, increased the expression of the downstream anti-apoptotic proteins B-cell CLL/lymphoma 2 (Bcl-2) and Bcl-xl, based on western blotting ($P < 0.05$). After treating B7-H3 overexpressing cells with the Jak2-specific inhibitor AG490, the phosphorylation of Jak2 and STAT3, and the expression of Bcl-2 and Bcl-xl, decreased accordingly ($P < 0.05$).

This finding suggested that the Jak2-STAT3 pathway is involved in the mechanism mediating the anti-apoptotic ability of B7-H3.

CONCLUSION: The overexpression of B7-H3 induces resistance to apoptosis in colorectal cancer cell lines by upregulating the Jak2-STAT3 signaling pathway, potentially providing new approaches to the treatment of colorectal cancer.

Key words: B7-H3; Overexpression; Colorectal cancer; B-cell CLL/lymphoma 2; Apoptosis; Signaling pathway; Jak2-STAT3

Core tip: The expression of B7-H3 has been positively correlated with poor prognosis in colorectal cancer. Previous studies revealed the relationship between B7-H3 and tumor invasion and metastasis. In the present study, the role of B7-H3 in apoptosis in colorectal cancer was investigated. Our results showed that overexpression of B7-H3 induced resistance to apoptosis in colorectal cancer cell lines by upregulating the Jak2-STAT3 signaling pathway. These results provide a new vision for designing therapeutics targeting B7-H3 and its associated signaling pathways in the treatment of colorectal cancer.

Zhang T, Jiang B, Zou ST, Liu F, Hua D. Overexpression of B7-H3 augments anti-apoptosis of colorectal cancer cells by Jak2-STAT3. *World J Gastroenterol* 2015; 21(6): 1804-1813 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i6/1804.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i6.1804

INTRODUCTION

B7-H3, first identified in 2001, is a member of the human B7 family of proteins, sharing 20%-27% amino acid identity with other B7 family members. As an important co-stimulatory molecule, B7-H3 promotes the proliferation of T cells and induces interferon (IFN)-γ production in the presence of T cell receptor signaling[1]. However, B7-H3 also acts as a T cell co-inhibitor. Most of the published data support the notion that B7-H3 inhibits T cell activation. Both mouse and human B7-H3 inhibit CD4 T cell activation and the production of effector cytokines such as IFN-γ and interleukin (IL)-4[2]. However, the function of B7-H3 in natural immunity and cancer immunity remains unclear.

The expression of B7-H3 in human tumor cells is positively correlated with the degree of disease malignancy, and B7-H3 participates in the process of tumor cell immune escape[3]. B7-H3 is highly expressed in many types of solid tumors, such as prostate cancer[4], pancreatic cancer[5], breast cancer[6], and gastric cancer[7]. Previous studies demonstrated a relationship between the expression of B7-H3 and poor prognosis in cancer patients[5,8,9]. The expression of B7-H3 is also closely related to colorectal cancer (CRC)[10]. Expression of B7-H3 not only has a negative relationship with prognosis in CRC[11] and the number of T cells in the tumor microenvironment[12] but also is positively correlated with invasion[13] and metastasis[14] in CRC.

The relationship between B7-H3 and the outcome of CRC cannot simply be explained by the regulation of B7-H3 in the immune system. The mechanism of abnormal B7-H3 expression in CRC and its role in the changes of tumor biological behavior need to be determined. Apoptosis, the process of programmed cell death, is an important field in tumor study[15]. However, few published papers have studied the relationship between B7-H3 and apoptosis, particularly in CRC. Therefore, we focused on the function of B7-H3 in apoptosis in CRC cells to discover the signal transduction pathway involved.

MATERIALS AND METHODS

Antibodies and reagents

Anti-human B7-H3, Bcl-2, Bcl-xl, Jak2, pJak2$^{Tyr1007/1008}$, STAT3, pSTAT3^{Tyr705}, and active caspase-3 antibodies were purchased from Abcam (Cambridge, MA, United States). An antibody against Bcl-2-associated X protein (Bax) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, United States). The horseradish peroxidase conjugated secondary anti-mouse and anti-rabbit antibodies and the GAPDH antibody were from Beyotime (Nantong, China). Tryphostins AG490 was from Sigma-Aldrich (St. Louis, MO, United States). The Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratory (Kumamoto, Japan).

Cells and cell culture

Two human CRC cell lines, SW620 and HCT8, exhibited different expression levels of B7-H3. We constructed SW620 cells that expressed high level of B7-H3 (SW620-B7-H3-EGFP), and HCT8 cells stably transfected with B7-H3 shRNA (HCT8-shB7-H3). Cells transfected with pIRES2-EGFP were used as negative controls (SW620-NC and HCT8-NC). All cells were cultured in Dulbecco's high glucose modified eagles medium (DMEM) (HyClone GE Healthcare Life Sciences, South Logan, UT, United States) supplemented with 10% fetal bovine serum at 37 ℃ in a humidified atmosphere with 5% CO_2. AG490, a Jak2 protein tyrosine kinase inhibitor, was dissolved in DMSO at a final concentration of 100 μmol/L. Clinical chemotherapeutics Oxaliplatin (L-OHP) and 5-fluorouracil (5-Fu) were used to detect the anti-apoptotic ability

of cancer cells.

RNA isolation, purification and first strand cDNA synthesis

Total RNA was isolated from 1.5 × 10^6 cells using TRIzol, following the manufacturer's instructions and quantified by a NanoDrop 2000 (Thermo Scientific, Waltham, MA, United States). Total RNA was treated with RNase-free DNase to remove residual genomic DNA. The first strand cDNA was synthesized from 1 μg RNA using an oligo-dT primer and AMV reverse transcriptase.

Relative real-time polymerase chain reaction

The expression levels of B7-H3, Bcl-2, Bcl-xl and Bax were analyzed relative to the level of the β-actin gene transcript using a Prism 7300 real-time polymerase chain reaction (PCR) instrument (Applied Biosystems Inc., Foster City, CA, United States). First-strand cDNA was amplified in a 20 μL PCR reaction mixture: 10 μL 2 × SYBR green PCR master mix, 0.4 μL 50 × ROX, 0.4 μL of each specific primer sets, and ddH_2O added to 20 μL. The sequences of primers were as follows: β-actin 5'- AGCGAGCATCCCCCAAAGTT-3' (sense), 5'-GGGCACGAAGGCTCATCATT-3' (antisense); B7-H3 5'-AGCACTGTGGTTCTGCCTCACA-3' (sense), 5'-CACCAGCTGTTTGGTATCTGTCAG-3' (antisense); Bcl-2 5'-CTGCACCTGACGCCCTTCACC-3' (sense), 5'-CACATGACCCCACCGAACTCAAAGA-3' (antisense); Bcl-xl 5'-GATCCCCATGGCAGCAGTAAAGCAAG-3' (sense), 5'-CCCCATCCCGGAAGAGTTCATTCACT-3' (antisense); Bax 5'-TCAACTGGGGCCGGGTTGTC-3' (sense), 5'-CCTGGTCTTGGATCCAGCC-3' (antisense). The PCR cycling consisted of 40 cycles of amplification of the cDNA with annealing at 60 ℃.

Western blotting

Western blotting was performed on whole-cell extracts prepared by lysing 1 × 10^6 cells in RIPA lysis buffer containing phosphatase inhibitor, protease inhibitor and 100 mmol/L PMSF (KeyGEN BioTECH, China) for 20 min on ice. The proteins were separated by 10% SDS-PAGE, except for active caspase-3 (15%), and then transferred onto a PVDF membrane (Merck Millipore, Germany). The membranes were blocked with 5% nonfat dry milk for 1 h at room temperature, and then incubated with the indicated antibodies at a concentration of 1:1000, except for Bax (1:100), at 4 ℃ overnight, followed by incubation with secondary antibody for 1 h at room temperature. The immunoreactive bands were visualized using Beyo ECL Plus (Beyotime, China).

Cell proliferation assay

Cells were seeded in 96-well plates at 5 × 10^3-8 × 10^3 cells/well and cultured overnight in DMEM. The next day, the medium was replaced with DMEM containing the different drugs with a two-fold concentration gradient. After 48 h, cell proliferation was quantified by a CCK-8 assay to calculate the inhibition rate. The assays were repeated at least three times.

Cell cycle and apoptosis assays

To analyze the effect of drugs on the different phases of the cell cycle, cells were incubated with different drug concentrations for 48 h. Cells harvested from each sample were then fixed with cold 70% ethanol at 4 ℃ overnight. The cells then were incubated with RNase A at 37 ℃ for 30 min and stained for 30 min in propidium iodide staining solution in the dark. Cell cycle analyses were performed with a FACScantoII flow cytometer and ModFit LT software (Verity Software House, ME, United States). For apoptosis analyses, we referred to the data of the sub-G1 peak.

Statistical analyses

Differences in mean values between groups were analyzed by a non-paired *t*-test. At least three independent experiments were performed for all the studies. Differences were considered to be statistically significant when P values were < 0.05. All of the data were analyzed using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, United States).

RESULTS

Overexpression of B7-H3 inhibited apoptosis

To investigate the relationship between B7-H3 and apoptosis in CRC cell lines, we performed western blotting with cell extracts from SW620-NC, SW620-B7-H3-EGFP, HCT8-NC and HCT8-shB7-H3 to demonstrate the expression of the apoptosis regulator proteins of the Bcl-2 family, including the anti-apoptotic proteins Bcl-2 and Bcl-xl and the pro-apoptotic protein Bax (Figure 1). Both B7-H3 overexpression in SW620 cells and downregulation in HCT8 cells affected the expression of anti- and pro-apoptotic proteins, at both the transcriptional and translational levels. In SW620-B7-H3-EGFP, the anti-apoptotic proteins Bcl-2 and Bcl-xl showed increased expression compared with SW620-NC ($P < 0.05$), while expression of the pro-apoptotic protein Bax decreased ($P < 0.05$). We observed a similar phenomenon in HCT8 cells ($P < 0.05$). The expressions of B7-H3 and the anti-apoptotic proteins were positively correlated in CRC cell lines. This suggested that the overexpression of B7-H3 might increase the resistance to apoptosis in tumor cells.

Overexpression of B7-H3 increased cell survival

To investigate whether B7-H3 altered the survival of CRC cells after chemotherapeutic treatment, we used a cell proliferation assay to detect the inhibition rate of SW620-NC, SW620-B7-H3-EGFP, HCT8-NC and HCT8-shB7-H3 treated with different concentrations of L-OHP and 5-Fu for 48 h (Figure 2). After treat-

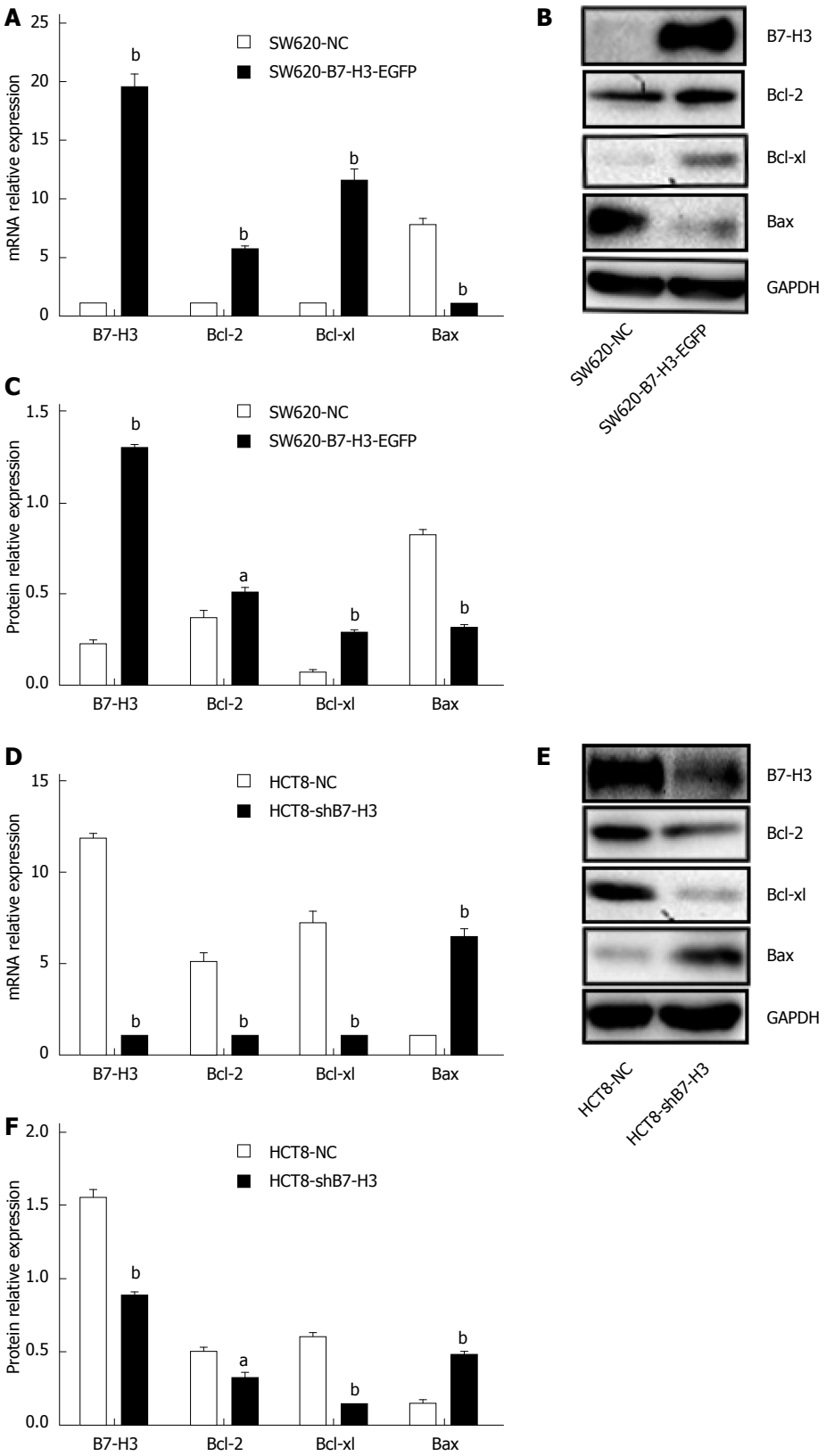


Figure 1 Overexpression of B7-H3 inhibits apoptosis. A: Real-time PCR for RNA levels of B7-H3, Bcl-2, Bcl-xl and Bax relative to β-actin in stably transfected SW620 cells, control cells (SW620-NC) and B7-H3 overexpressing cells (SW620-B7-H3-EGFP); B: Western blot analysis for B7-H3, Bcl-2, Bcl-xl, Bax and GAPDH protein levels in whole-cell lysates from the SW620 cells; C: Comparison of relative protein levels between the SW620 cells from (B); D: Real-time PCR for RNA levels of B7-H3, Bcl-2, Bcl-xl and Bax relative to β-actin in stably transfected HCT8 cell lines, the control cells (HCT8-NC) and the B7-H3 knockdown cells (HCT8-shB7-H3); E: Western blot analysis for B7-H3, Bcl-2, Bcl-xl, Bax and GAPDH protein levels in whole-cell lysates from the HCT8 cells; F: Comparison of relative protein levels between the HCT8 cells from (E). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control. Bax: Bcl-2-associated X protein; Bcl-2: B-cell CLL/lymphoma 2; Bcl-xl: B-cell lymphoma-extra large; NC: Negative control.

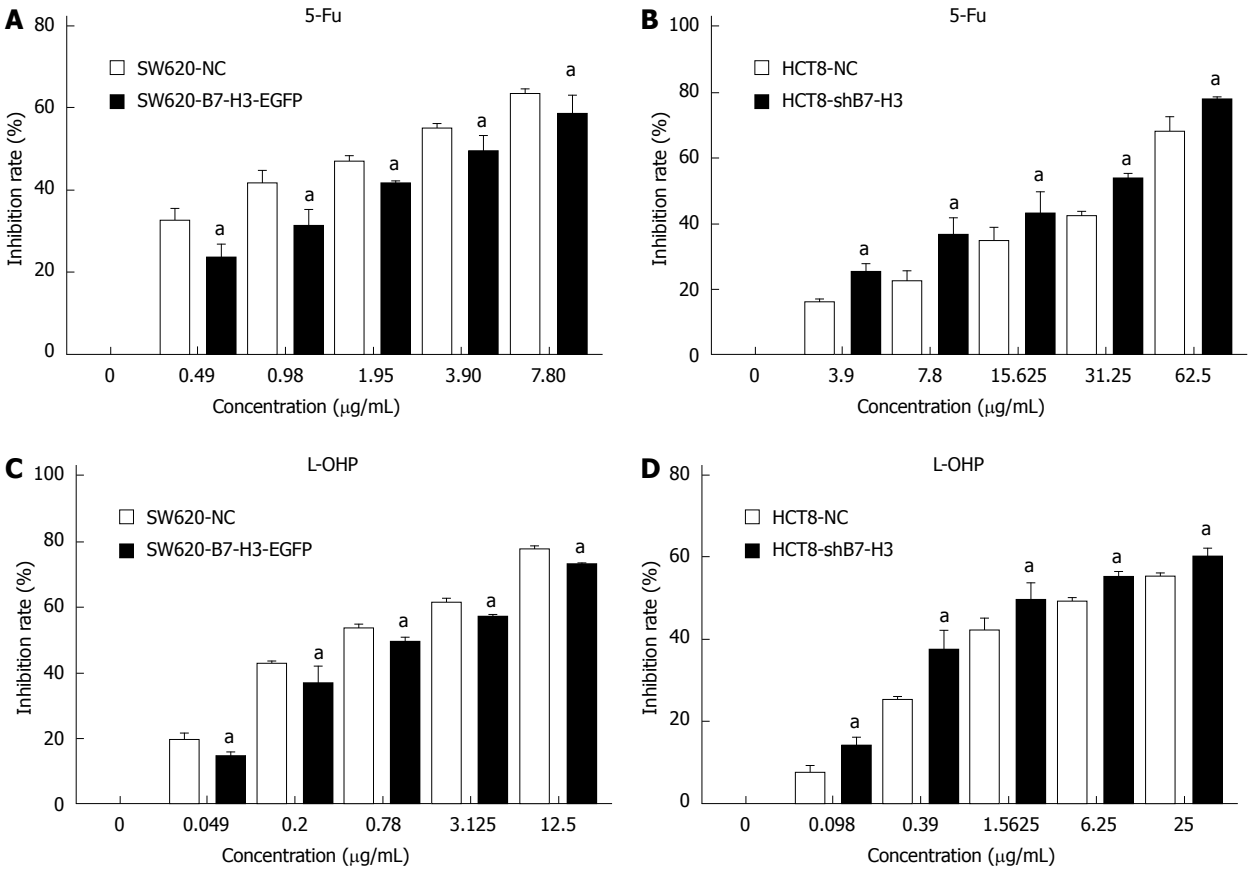


Figure 2 Overexpression of B7-H3 increases cell survival. Stably transfected SW620 and HCT8 cell lines were incubated with 5-Fu or L-OHP for 48 h. A CCK-8 assay was used to detect the inhibition rate of cells treated with different concentration of different drugs. A: The control cells (SW620-NC) and the B7-H3 overexpressing cells (SW620-B7-H3-EGFP) were incubated with 5-Fu; B: The control cells (HCT8-NC) and the B7-H3 knockdown cells (HCT8-shB7-H3) were incubated with 5-Fu; C: SW620-NC and SW620-B7-H3-EGFP were incubated with L-OHP; D: HCT8-NC and HCT8-shB7-H3 were incubated with L-OHP. $^{a}P < 0.05$ *vs* control. 5-Fu: 5-Fluorouracil; L-OHP: Oxaliplatin; NC: Negative control.

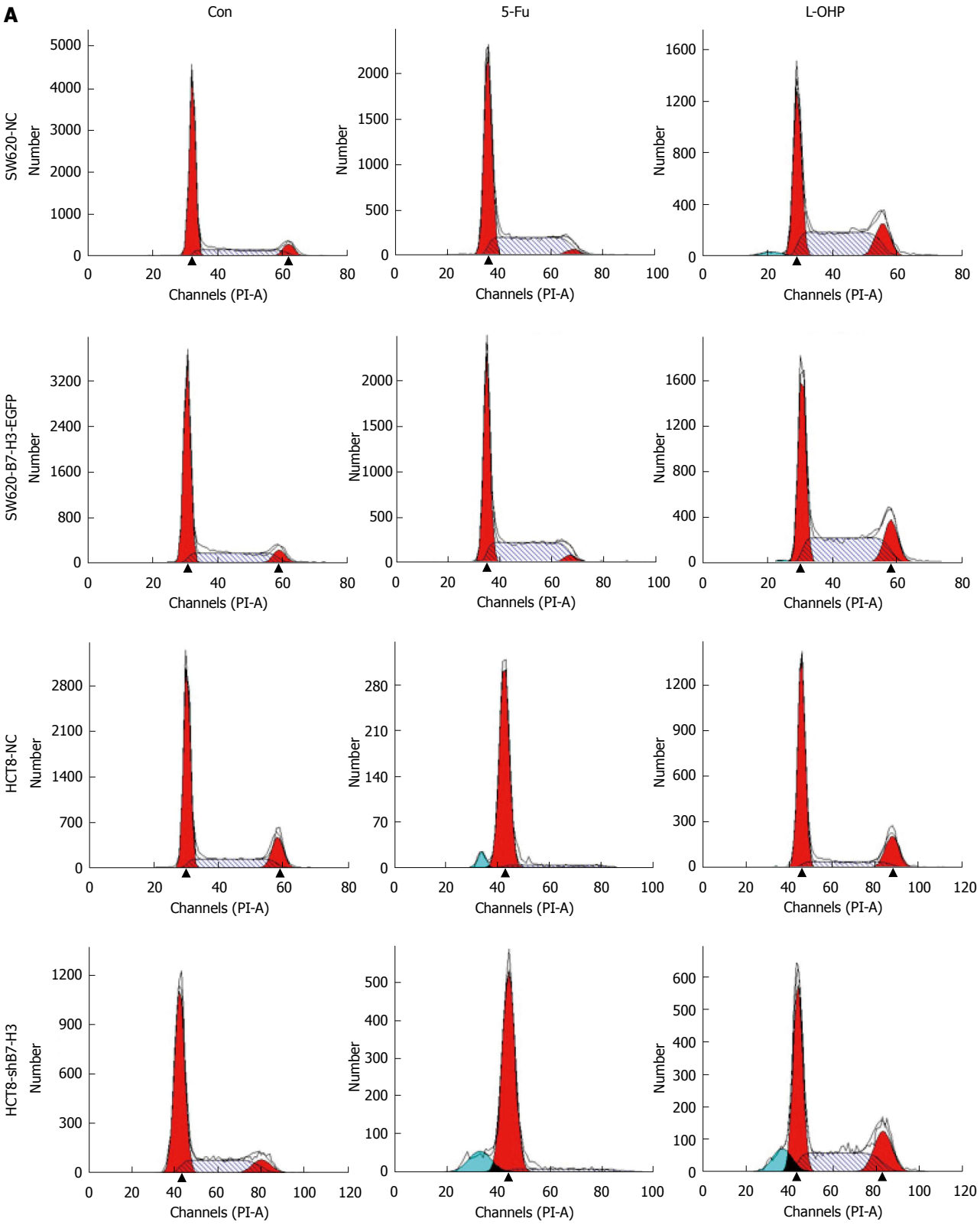
ment with L-OHP or 5-Fu at any concentration, the inhibition rate of SW620-B7-H3-EGFP was less than that of SW620-NC ($P < 0.05$). The HCT8 cells showed similar results ($P < 0.05$). Therefore, we hypothesized that overexpression of B7-H3 increased the cells' resistance to drugs, resulting in a higher survival rate in the cells that overexpressed B7-H3.

Overexpression of B7-H3 suppressed the apoptotic ability of CRC cells by weakening their sensitivity to drugs

We described the increased anti-apoptotic effect in the B7-H3 overexpressing cells above. To investigate the exact response to apoptosis resulting from chemotherapeutic treatment, we treated SW620-NC, SW620-B7-H3-EGFP, HCT8-NC and HCT8-shB7-H3 cells with a high concentration (50 μg/mL) of 5-Fu or L-OHP for 48 h. A cell cycle assay was used to detect the rate of apoptosis in each cell line, according to the sub-G1 peak (Figure 3A and B). We found that SW620-B7-H3-EGFP had stronger resistance to 5-Fu or L-OHP and less apoptosis compared with SW620-NC ($P < 0.05$). Similar results were found in the HCT8 cells ($P < 0.01$). We used western blotting to detect active caspase-3 and compare apoptosis rates pairwise (Figure 3C). Similarly, the expression of active caspase-3 in B7-H3 overexpressing cells was less than that seen in cells with downregulated B7-H3. We concluded that overexpression of B7-H3 could inhibit apoptosis in CRC cell lines.

Overexpression of B7-H3 enhanced the anti-apoptotic effect in CRC cells via activation of Jak2-STAT3 pathway

We observed chemoresistance accompanied by decreased apoptosis in 5-Fu or L-OHP treated B7-H3 overexpressing cells; therefore, we investigated which signaling pathway was involved in the apoptotic response. The Jak2-STAT3 pathway was reported to regulate anti-apoptotic molecules downstream of B7-H3 in breast cancer cells[16]. As can be seen in Figure 1A, the overexpression of B7-H3 indeed upregulated the expression of anti-apoptotic proteins. We therefore asked whether the Jak2-STAT3 pathway played the same role in CRC cells. We treated SW620-B7-H3-EGFP cells with the Jak2-specific inhibitor AG490 for 24 h at final concentration of 1 μmol/L. We performed western blotting with whole-cell lysates from SW620 cells to detect the expression of Jak2, STAT3 and their phosphorylated forms. To investigate the involvement of the Jak2-


A
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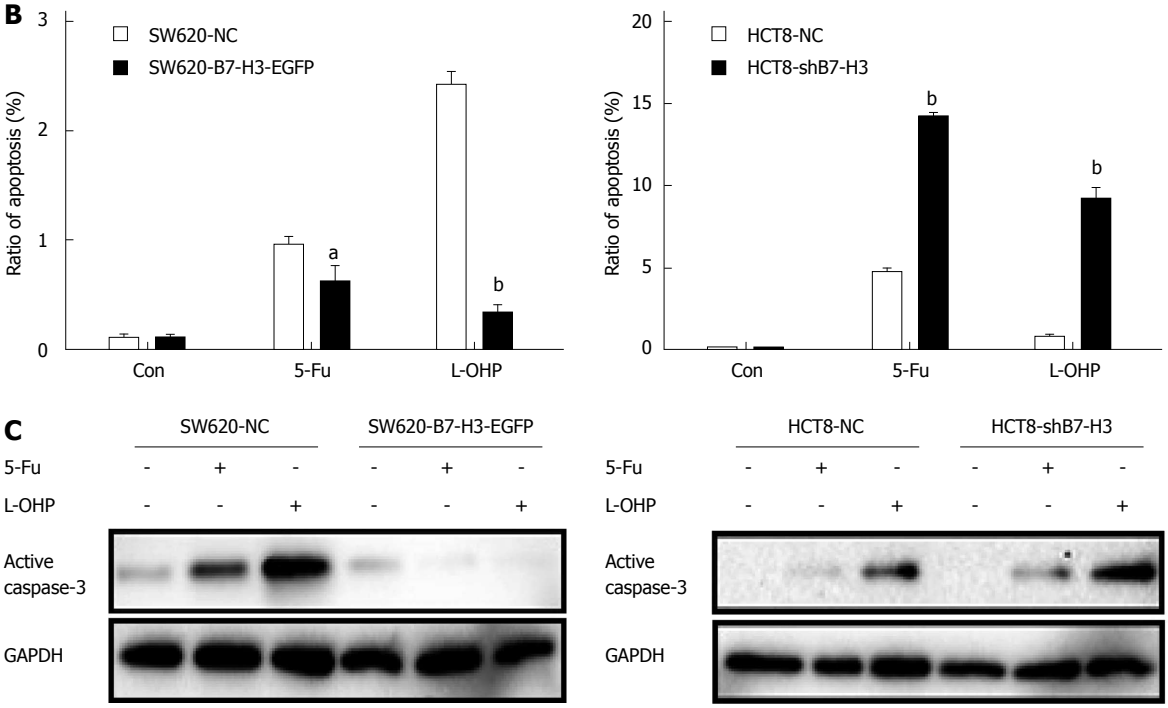

Figure 3 Overexpression of B7-H3 suppresses apoptosis in colorectal cancer cells by weakening the sensitivity to drugs. Stably transfected SW620 and HCT8 cell lines were incubated with a high concentration of 5-Fu or L-OHP (50 μg/mL) for 48 h. A: The ratio of apoptosis was detected by cell cycle assay according to sub-G1 peak; B: Statistical results were used to analyze the rate of apoptosis from (A); C: Western blot analysis for active Caspase-3 and GAPDH protein levels in whole-cell lysates from stably transfected SW620 and HCT8 cell lines. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control. 5-Fu: 5-Fluorouracil; L-OHP: Oxaliplatin.

STAT3 pathway in the anti-apoptotic effect of B7-H3, we also assayed the expression of downstream apoptotic regulator proteins by western blotting (Figure 4). We observed that the phosphorylation levels of Jak2 and STAT3 increased following B7-H3 overexpression ($P < 0.05$). After AG490 treatment, the phosphorylation level of STAT3 was almost abolished because of the inhibition of Jak2 tyrosine phosphorylation ($P < 0.05$). This result indicated that the effect of B7-H3 on STAT3 occurred through Jak2. The expression of the anti-apoptotic proteins Bcl-2 and Bcl-xl decreased with reduced expression of phosphorylation level of Jak2 and STAT3 ($P < 0.05$), and the expression of the pro-apoptotic protein Bax increased correspondingly ($P < 0.05$). In summary, these results suggested that B7-H3 overexpression increased Jak2 phosphorylation, leading to higher STAT3 phosphorylation, which, in turn, led to the increased expression of the anti-apoptotic proteins Bcl-2 and Bcl-xl. Thus, we confirmed that the Jak2-STAT3 signaling pathway played an important role in regulating the anti-apoptotic ability of B7-H3.

DISCUSSION

In this study, the role of B7-H3 in apoptosis in colorectal cancer cell lines was investigated. Overexpression of B7-H3 increased the anti-apoptotic ability and resistance to chemotherapeutics, whereas knockdown of B7-H3 led to increased sensitivity to drug-induced apoptosis. Furthermore, we proved that B7-H3 regulated the expression of Bcl-2, Bcl-xl and Bax *via* the Jak2-STAT3 signaling pathway to affect the anti-apoptotic ability of cancer cells.

First, we examined the B7-H3 expression level in CRC cell lines. B7-H3 was expressed at different levels in all cell lines. SW620 had lower B7-H3 expression, while HCT8 had higher expression. SW620 was therefore chosen to generate a B7-H3 upregulated stably transfected derivative cell line, and HCT8 was chosen to construct a B7-H3 downregulated stably transfected derivative cell line. Four stably transfected CRC cell lines were generated: the B7-H3 overexpressing cell line SW620-B7-H3-EGFP, the B7-H3 knockdown cell line HCT8-shB7-H3, and the control cell lines SW620-NC and HCT8-NC.

The apoptosis regulator Bcl-2 is a member of a family of evolutionarily related proteins. These proteins can be either pro-apoptotic (including Bax, Bad, Bak and Bok) or anti-apoptotic (including Bcl-2 proper, Bcl-xl, and Bcl-w). Bcl-2 is one of the most important oncogenes in apoptosis research[17,18]. In our study, overexpression of B7-H3 caused the CRC cells to be more resistant to apoptosis because of the upregulation of Bcl-2 and Bcl-xl and the downregulation of Bax, thus reducing the sensitivity of cells to chemotherapeutics. In contrast, knockdown of B7-H3 increased drug-induced apoptosis. This is consistent with Zhao's investigation, in which silencing of B7-H3 increased the sensitivity of the human pancreatic carcinoma cell line Patu8988 to gemcitabine as a result of enhanced drug-induced apoptosis[19]. This sug-

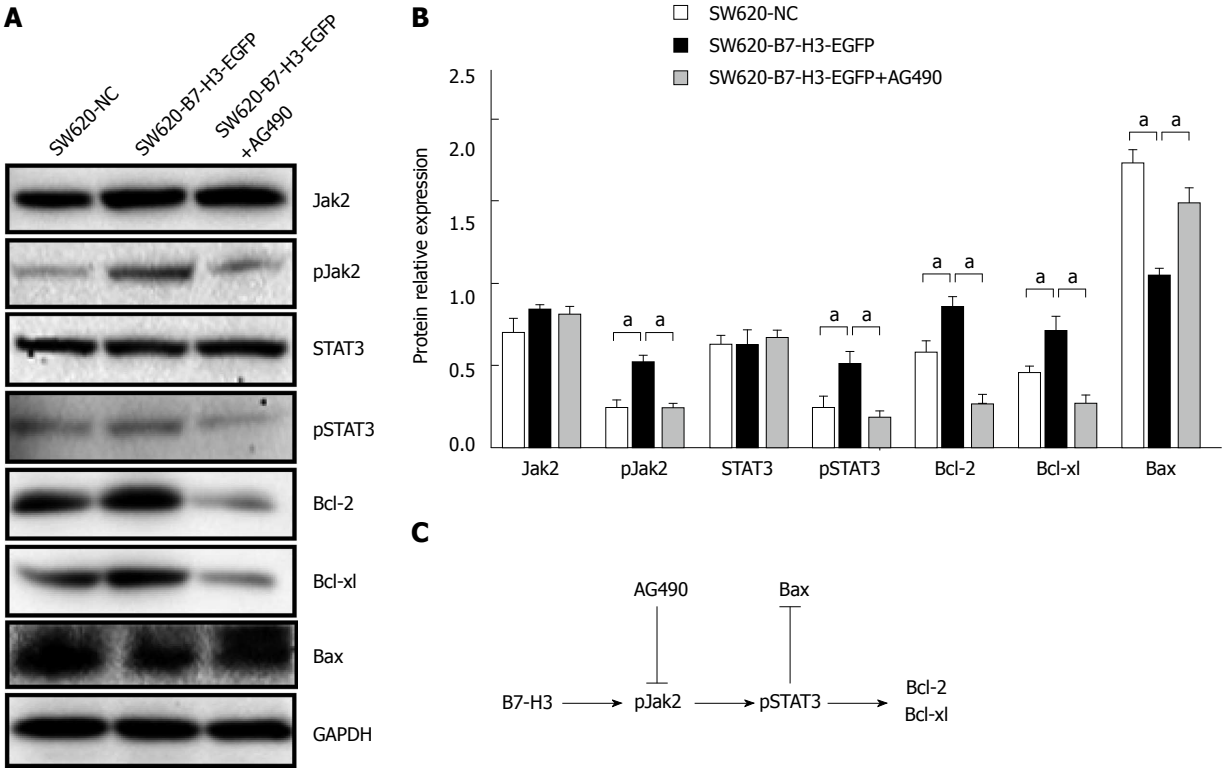

Figure 4 Overexpression of B7-H3 enhances the anti-apoptotic effect in colorectal cancer cells *via* the activation of the Jak2-STAT3 pathway. A: Western blot analysis with the control cells (SW620-NC), the B7-H3 overexpressing cells (SW620-B7-H3-EGFP) and the AG490 treated B7-H3 overexpressing cells (SW620-B7-H3-EGFP+AG490) demonstrating the expression of Jak2-STAT3 pathway proteins and apoptosis regulator proteins; B: Comparison of relative protein levels from (A); C: A simple pathway map for B7-H3 regulation of the anti-apoptotic effect in CRC cells. $^{a}P < 0.05$ *vs* control. CRC: Colorectal cancer; Jak2: Janus kinase 2; NC: Negative control; STAT3: Signal transducer and activator of transcription 3.

gested that overexpression of B7-H3 in CRC patients made them inappropriate candidates for treatment with chemotherapeutics. This may also explain why the expression of B7-H3 is associated with poor prognosis in CRC[11]. It may be important to downregulate the expression of B7-H3 in patients for them to benefit from drug-induced apoptosis.

STAT3 is a transcription factor that mediates the expression of a variety of genes in response to cell stimuli[20,21], and thus plays a key role in many cellular processes, such as cell growth[22] and apoptosis[23]. It is activated through phosphorylation by the non-receptor tyrosine kinase Jak2, and high activity has been shown to predict resistance to chemotherapeutics resulting from the upregulation of anti-apoptotic proteins[24]. Jak2-STAT3 signaling, while regulating many aspects of cancer development and progression, promotes invasion and metastasis[25]. Interestingly, we found that upregulation of B7-H3 activated the phosphorylation of both Jak2 and STAT3, which led to the increased expression of Bcl-2 and Bcl-xl. This may explain why B7-H3 overexpressing cells were more resistant to drug-induced apoptosis. In a previous study, Liu *et al*[16] reported a similar finding in breast cancer. In that study, they only generated a B7-H3 knockdown model, and lacked a functional recruitment experiment. Our results confirmed the relationship between B7-H3 and the Jak2-STAT3 signal transduction pathway by both B7-H3 downregulation and overexpression models. Furthermore, the blockage of Jak2 phosphorylation by its specific inhibitor AG490 resulted in a reduction in STAT3 phosphorylation and expression of anti-apoptotic proteins in B7-H3 overexpressing cells. AG490, a specific Jak2 inhibitor, could block B7-H3 regulation of apoptosis related proteins, providing more insight into Jak2-STAT3 signal transduction and B7-H3.

A great deal of evidence exists that reinforces the link between inflammation and colorectal cancer[26-28]. The molecular pathobiology of CRC implicates pro-inflammatory conditions in promoting the progression of tumor malignancy, invasion and metastasis[29]. Patients with inflammatory bowel disease are at higher risk of CRC[30]. Furthermore, the Jak2-STAT3 signaling pathway mediates the progression of inflammation[31,32]. Activators of the Jak2-STAT3 signaling pathway are important factors released during inflammation[33]. In particular, anti-inflammatory cytokines such as IL-10 activate STAT3 phosphorylation *via* Jak2[34]. Oxidative stress and cytokines such as IL-6 also activate STAT3 by a Jak2-dependent mechanism[35]. The primary consequence of the activation of this pathway is to promote inflammation-associated gene expression; however, pathway activation also regulates survival-associated gene expression[36]. The role of B7-H3 overexpression in tumor cells in activating the Jak2-

STAT3 signaling pathway augmented the activation of inflammation by Jak2-STAT3. This partially explained the reports of the negative relationship between B7-H3 and prognosis of CRC.

In summary, our study investigated the impact of the overexpression of B7-H3 in resistance to apoptosis mediated by the Jak2-STAT3 signaling pathway. We focused on the non-immunological function of B7-H3 in CRC. These results suggest that new CRC treatments could target B7-H3 overexpression or associated signaling pathways in tumors as a novel approach to weaken drug resistance.

COMMENTS

Background

Expression of B7-H3 has been positively correlated with poor prognosis in colorectal cancer (CRC). Previous studies have revealed the relationship between B7-H3 and tumor invasion and metastasis. However, the function of B7-H3 in apoptosis and the molecular mechanism involved in remains obscure.

Research frontiers

This study was performed to explore the role of B7-H3 in apoptosis in CRC cell lines through cellular and molecular biological methods. CRC cell lines that either up- or downregulated B7-H3 expression were constructed to detect the related indicators of apoptosis, such as the expression of apoptosis regulator proteins, the cell cycle and the expression of active Caspase-3 with drug treatment. Furthermore, the molecular mechanism of B7-H3 in regulating apoptosis was also discussed in detail.

Innovations and breakthroughs

This study showed that overexpression of B7-H3 induces resistance to apoptosis in issue 7 cell lines by upregulating the Jak2-STAT3 signaling pathway.

Applications

These results provide a new paradigm for designing treatment strategies targeting B7-H3 and signaling pathways in CRC treatment.

Peer-review

This study provides novel and interesting insights into the function of B7-H3 on tumor cells in addition to its co-inhibitory role in T cell activation.

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ORIGINAL ARTICLE

Basic Study

Overexpression of Csk-binding protein decreases growth, invasion, and migration of esophageal carcinoma cells by controlling Src activation

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Abstract

AIM: To investigate the mechanisms by which Csk-binding protein (CBP) inhibits tumor progression in esophageal carcinoma.

METHODS: A CBP overexpressing esophageal carcinoma cell line (TE-1) was established. The growth, invasion, and migration of CBP-TE-1 cells, as well as the expression of Src were then determined and compared with those in normal TE-1 cells.

RESULTS: The expression of Src was decreased by the overexpression of CBP in TE-1 cells. The growth, invasion, and migration of TE-1 cells were decreased by the overexpression of CBP.

CONCLUSION: This study indicates that CBP may decrease the metastasis of esophageal carcinoma by inhibiting the activation of Src. CBP may be a potential tumor suppressor and targeting the *CBP* gene may be an alternative strategy for the development of therapies for esophageal carcinoma.

Key words: Csk-binding protein; Esophageal carcinoma; Cell growth; Invasion; Migration

Core tip: Csk-binding protein (CBP) is a ubiquitously expressed transmembrane protein and functions as a suppressor of Src-mediated tumor progression by promoting the inactivation of Src. Here, we established a CBP overexpressing esophageal carcinoma cell line

(TE-1) and found that the overexpression of CBP significantly decreased the proliferation, invasion, and migration of TE-1 cells, accompanied by decreased activation of Src. These results indicate that CBP may decrease the metastasis of esophageal carcinoma by inhibiting the activation of Src. Targeting the *CBP* gene may be an alternative strategy for the development of therapies for esophageal carcinoma.

Zhou D, Dong P, Li YM, Guo FC, Zhang AP, Song RZ, Zhang YM, Li ZY, Yuan D, Yang C. Overexpression of Csk-binding protein decreases growth, invasion, and migration of esophageal carcinoma cells by controlling Src activation. *World J Gastroenterol* 2015; 21(6): 1814-1820 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i6/1814.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i6.1814

INTRODUCTION

Csk-binding protein (CBP), a ubiquitously expressed transmembrane protein, functions as a suppressor of Src-mediated tumor progression by promoting the inactivation of Src[1,2]. CBP is widely reported to act as a scaffold in the Csk-mediated negative regulation of Src family kinases (SFKs)[3,4]. First, CBP is phosphorylated by SFKs, then it associates with C-terminal Src kinase (Csk) through a specific site (Tyr-317 in humans) and brings it into proximity with membrane-associated SFKs. After that, Csk phosphorylates the C-terminal negative regulatory tyrosine residue of SFKs, which suppresses their activation[1]. SFKs are membrane-associated non-receptor protein tyrosine kinases that play pivotal roles in regulating various cellular processes including proliferation, differentiation, adhesion, migration, and survival[5]. Thus, CBP plays the opposite role in various Csk-mediated cellular processes and might be a significant target for Csk-mediated tumors.

Recent studies have shown that CBP is expressed at low levels in various human cancer cells[6-8], suggesting that CBP may be an important suppressor in the progression of various human cancers. We have previously reported that the expression of CBP is markedly down-regulated in esophageal carcinoma[9]; however, the mechanisms by which the down-regulation of CBP affects the progression of esophageal carcinoma remain unknown. Therefore, we established an esophageal carcinoma cell line stably overexpressing CBP (TE-1). We found that overexpression of CBP decreased the growth, invasion, and migration of esophageal carcinoma cells.

MATERIALS AND METHODS

Cell culture

The human esophageal carcinoma cell line TE-1 was provided by the Cell Bank of the Chinese Academy of Sciences, Shanghai, China. Cells were cultured in RPM1640 medium supplemented with 10% fetal bovine serum.

Lentiviral vector constructs and preparation

A lentiviral-delivered CBP vector was constructed and prepared by Shanghai qcbio Science & Technologies Co., Ltd. (Shanghai, China), as described by Lois *et al*[10]. Briefly, primers were designed according to the CBP sequence (Genbank Accession Number NC_000008.10). The primer sequences were: CBP-F, 5′-GGAATTCCCTGCCATGGGGCCCGCG-3′; CBP-R, 5′-GGAATTCGAGCCTGGT AATATCTCTGCCT-3′. The target gene was obtained by polymerase chain reaction and was inserted into the pUC57 vector. Subsequently, both pLenO-DCE and pUC57-CBP were digested by EcoRI and NotI. After ligation, the pLenO-DCE-CBP vector was constructed. After sequencing, the pLenO-DCE-CBP vector was transfected into 293T cells and the lentiviral-delivered CBP vector was prepared.

Cell transfection

Briefly, 1 × 10^6 TE-1 cells were seeded in each well of a 6-well plate in 500 μL of complete medium at 37 ℃ in a 5% CO_2 incubator for 24 h, and then transduced by lentiviral vectors at a multiplicity of infection of 10:1[11]. Transduction was carried out in the presence of polybrene (8 μg/mL). After washing three times with PBS, 1 mL of RPMI1640 was added in each well. Cells were seeded at 37 ℃ in a 5% CO_2 incubator for 48 h. Fluorescence microscopy was used to observe the transduction. G418 (400 μg/mL) was used for screening. Transduced cells were passaged and seeded for further experiments. Also, the pLenO-DCE-(-) vector was transduced into TE-1 cell as a blank transfection control.

MTT assay

Cells (5 × 10^3) were seeded in a 96-well plate (BD Biosciences, United States) and harvested for the MTT assay at different time points from days 1-6. Cell samples were incubated with 20 μL of MTT (5 mg/mL; Sigma, United States) for 6 h. Following the removal of the MTT solution, formazan crystals were dissolved in 150 μL of dimethyl sulfoxide (DMSO, Sigma, United States). The absorption of the solution was measured at 570 nm[12].

Transwell invasion assay

Invasion chambers coated with Matrigel were purchased from BD Biosciences. Assays were conducted as described by Seton-Rogers *et al*[13]. Briefly, cells (1 × 10^5) were added to the top chambers (in 300 μL of RPMI1640) of 24-well Transwell plates (BD Biosciences; 8 μm pore size). After 24 h, the top (non-migrated) cells were removed, and the bottom (migrated) cells were fixed with 70% methanol and stained with trypan

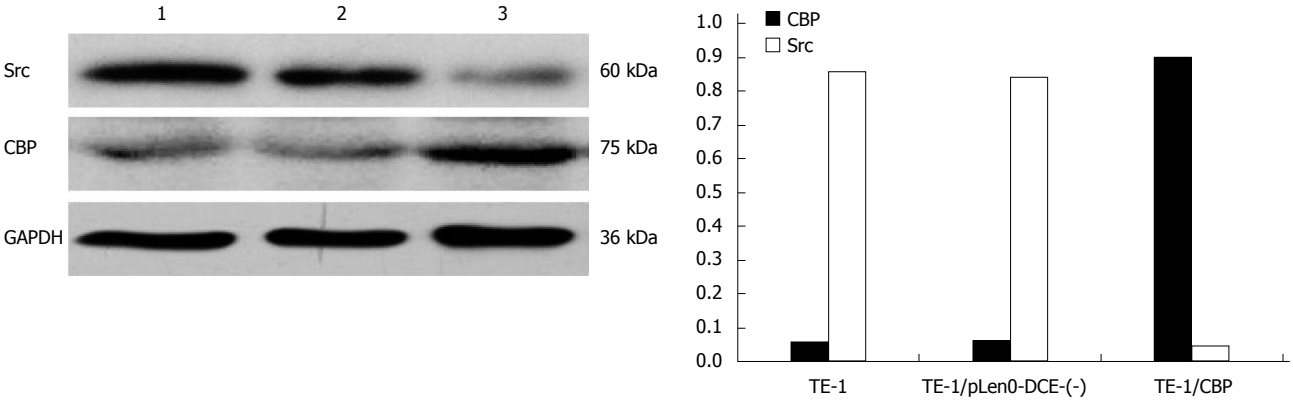

Figure 1 Overexpression of Csk-binding protein decreases the expression of Src in TE-1 cells. Western blot analysis of the expression of Csk-binding protein (CBP) and Src in TE-1 cells before and after transduction with CBP lentiviral vectors. GAPDH was used as a protein loading control. The expression of CBP was much higher in transduced TE-1 cells than in normal TE-1 and TE-1/pLenO-DCE cells ($P < 0.05$), and expression of Src was much lower in transduced TE-1 cells than in normal TE-1 and TE-1/pLenO-DCE cells ($P < 0.05$). 1 = normal TE-1 cells; 2 = TE-1 cells after transfection with pLenO-DCE-(-); 3 = TE-1 cells after overexpression CBP.

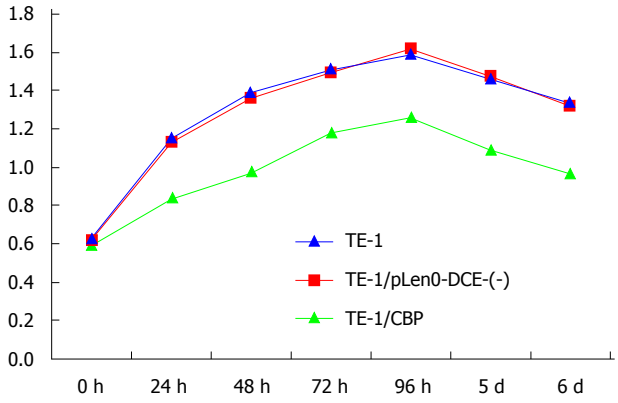

Figure 2 Cell growth of TE-1, TE-1/pLenO-DCE-(-) and TE-1/Csk-binding protein cells. The growth of TE-1/CBP cells was slower than that of TE-1 and TE-1/pLenO-DCE-(-) cells ($P < 0.05$).

blue to visualize nuclei. The number of migrating cells in five fields was counted at 100× magnification, and the mean for each chamber was determined with ImageJ (version 1.38, National Institutes of Health). Experiments were repeated a minimum of three times.

Scratch assay

Scratch assay was used for the detection of cell migration as described by Gough *et al*[14]. Briefly, cells (1×10^6) were seeded in a 6-well plate (BD Biosciences, United States) until cells reached 100% confluence. A p200 pipette tip was then used to create a scratch of the cell monolayer. After washing the plate once with PBS and replacing with the new medium, the cells were incubated at 37 ℃ in a 5% CO_2 incubator. After 24 h, the number of cells that had migrated into the scratch was calculated at 100× magnification.

Western blot analysis

Cells were lysed on ice in RIPA buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 2 mmol/L sodium fluoride, 2 mmol/L Na_3VO_4, 1 mmol/L EDTA, and 1 mmol/L EGTA). Total protein extracts were analyzed by Western blot, as described previously[15]. Proteins (20 μg) were separated by SDS-PAGE (Invitrogen) and transferred to PVDF membranes. The membranes were blotted for 1 h with 5% milk. Membranes were incubated with a primary antibody (1:500 dilution) against CBP or Src (Santa Cruz Biotechnology, Inc., United States) at 4 ℃ overnight. After incubation with a horseradish peroxidase-conjugated secondary antibody (1:1000 dilution) for 3 h at 37 ℃, signals were detected by ECL chemiluminescence for 5 min. The films were analyzed by densitometry with image software.

Statistics analysis

Data are expressed as mean ± SE and were statistically evaluated by one-way ANOVA followed by a Newman-Keuls test. $P < 0.05$ was considered statistically significant.

RESULTS

CBP overexpression decreases the expression of Src in TE-1 esophageal carcinoma cells

To check the overexpression of CBP in our stably transfected cells and to determine if overexpression of CBP leads to decreased expression of Src, we assessed the expression of Src in our TE-1 esophageal carcinoma cells that had been transduced with lentiviral constructs to overexpress CBP. As illustrated in Figure 1, our stably transduced cells overexpressed CBP, and CBP overexpression significantly decreased the protein levels of Src ($P < 0.05$) (Figure 1), supporting the previous finding that CBP down-regulates the activity

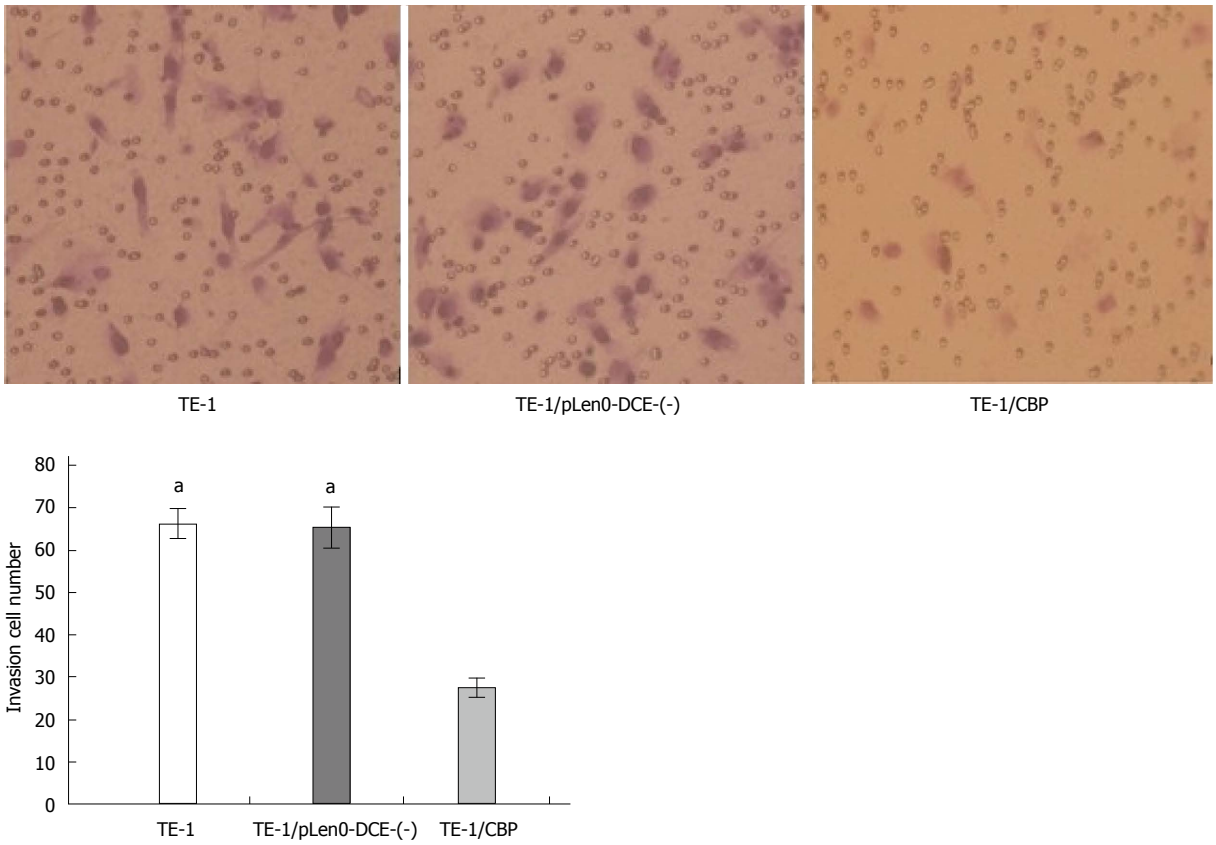

Figure 3 Invasion of TE-1, TE-1/pLenO-DCE-(-) and TE-1/Csk-binding protein cells. Trypan blue staining showed that TE-1, TE-1/pLenO-DCE-(-) and TE-1/CBP cells passed through the Matrigel (200×). [a]*P* < 0.05 *vs* TE-1/CBP cells, no significant difference compared with TE-1/pLenO-DCE-(-) cells.

of Src[1,2].

Overexpression of CBP inhibits the growth of TE-1 esophageal carcinoma cells

We assessed the cell growth of TE-1, pLenO-DCE-(-) vector transfected TE-1 (TE-1/pLenO-DCE) and CBP overexpressing TE-1 (TE-1/CBP) cells. As illustrated in Figure 2, compared with TE-1 and TE-1/pLenO-DCE cells, the cell growth of TE-1/CBP cells decreased significantly ($P < 0.05$) (Figure 2).

Overexpression of CBP decreases the invasion of TE-1 esophageal carcinoma cells

As illustrated in Figure 3, Transwell analysis showed that the numbers of TE-1 cells (62.2 ± 3.6) and TE-1/pLenO-DCE cells (65.4 ± 4.8) passing through the Matrigel were markedly higher than that of TE-1/CBP cells (27.6 ± 2.2) ($P < 0.05$) (Figure 3).

Overexpression of CBP decreases the migration of TE-1 esophageal carcinoma cells

As illustrated in Figure 4, scratch analysis showed that the distance of TE-1 cells (116.2 ± 6.4 μm) and TE-1/pLenO-DCE cells (122.4 ± 8.8 μm) migrating into the scratch was markedly greater than that of TE-1/CBP cells (66.6 ± 6.2 μm) ($P < 0.05$) (Figure 3).

DISCUSSION

CBP is weakly expressed in many types of human cancers, including non-small cell lung cancer[7], esophageal cancer[9], and head and neck squamous cell cancer[16], suggesting that CBP may play an important suppressive role in the progression, development, and invasion of cancers. Recently, we reported that the expression of CBP is down-regulated in esophageal carcinoma[9], but the functions of CBP in esophageal carcinoma remain undetermined. To investigate the role of CBP in esophageal carcinoma, we examined the alteration of cell growth, invasion and migration of esophageal carcinoma cells after overexpressing CBP.

We up-regulated the expression of CBP by transfection of TE-1 esophageal carcinoma cells with CBP constructs. Our data demonstrate that CBP overexpression resulted in a decrease in the growth, invasion, and migration of TE-1 cells. These findings indicate that CBP plays an important role in the suppression of tumor progression. Since tumor progression inhibition is a prerequisite for efficient tumor therapy[17,18], CBP might be a promising target for tumor therapy. Our data also indicate that controlling the activation of the Src pathway may be one of the underlying mechanisms by which CBP up-regulates

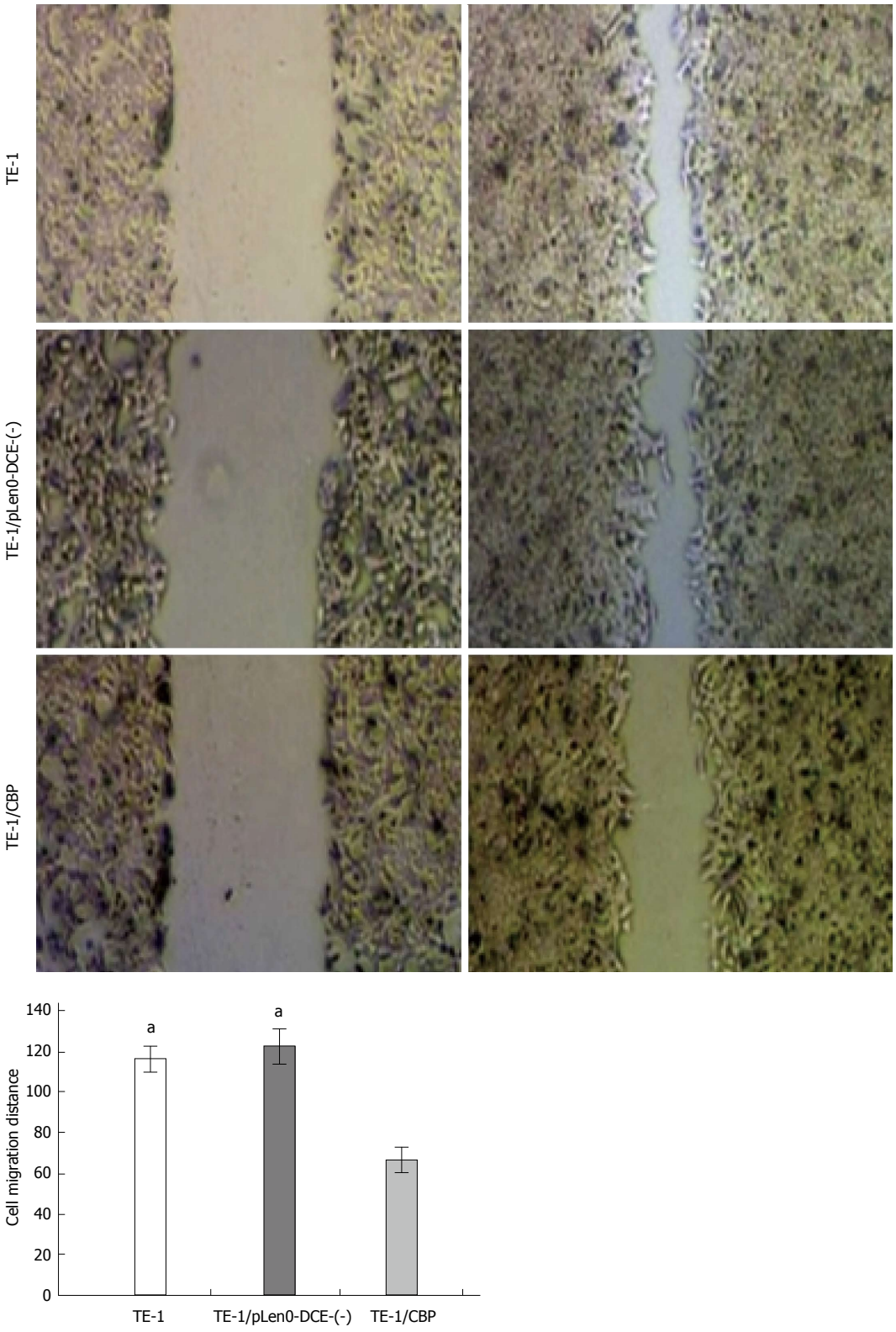

Figure 4 Migration of TE-1, TE-1/pLenO-DCE-(-) and TE-1/ Csk-binding protein cells. The scratch assay showed that TE-1, TE-1/pLenO-DCE-(-) and TE-1/CBP cells moved into the scratch (200×). The distance of cell migration was assessed. $^{a}P < 0.05$ *vs* TE-1/CBP cells, no significant difference compared with TE-1/pLenO-DCE-(-) cells.

the growth, invasion and migration of esophageal carcinoma cells.

Src signaling, a prominent cancer cell growth-promoting and invasion-promoting pathway[19,20], can be activated by receptor tyrosine kinases and integrins to promote the growth, migration, and invasion of tumor cells. Although many studies have demonstrated the role of CBP in promoting the inactivation of Src

signaling[1,2], we sought to investigate whether the up-regulation of CBP is able to decrease the growth, invasion, and migration of esophageal carcinoma cells through the inactivation of Src signaling. We found that CBP overexpression decreased the activity of Src in TE-1 esophageal carcinoma cells, and reduced esophageal carcinoma cell growth, invasion, and migration. Thus, we propose that CBP up-regulation decreases esophageal carcinoma progression, at least partially, through the inactivation of Src signaling. Further studies are needed to determine the molecular mechanism(s) by which CBP regulates Src signaling.

In summary, we demonstrated that CBP inhibits the growth, invasion, and migration of esophageal carcinoma cells by inactivating Src signaling, suggesting that CBP plays an important role in the inhibition of tumor progression and development of esophageal carcinoma. CBP as an effective target aiming to modulate Src signaling may be a reasonable approach to treating esophageal carcinoma.

COMMENTS

Background

Csk-binding protein (CBP) is a ubiquitously expressed transmembrane protein and functions as a suppressor of Src-mediated tumor progression by promoting the inactivation of Src. The authors previously reported that expression of CBP is markedly down-regulated in esophageal carcinoma; however, the mechanisms by which the down-regulation of CBP affects the progression of esophageal carcinoma remain unknown.

Research frontiers

Previous studies have suggested that CBP has an opposing role in various Csk-mediated cellular processes and might be a significant target in Csk-mediated tumors. CBP is weakly expressed in many types of human cancers, including non-small cell lung cancer, esophageal cancer, and head and neck squamous cell cancer, suggesting that CBP may play an important suppressive role in the progression, development, and invasion of cancers.

Innovations and breakthroughs

The authors have reported that the expression of CBP is down-regulated in esophageal carcinoma, but the functions of CBP in esophageal carcinoma remain to be determined. To investigate the role of CBP in esophageal carcinoma, the authors examined the alterations in the growth, invasion, and migration of esophageal carcinoma cells overexpressing CBP. The data demonstrate that CBP overexpression resulted in a decrease in the growth, invasion, and migration of TE-1 cells. These findings indicate that CBP plays an important role in suppressing tumor progression.

Applications

This study indicates that CBP plays an important role in the inhibition of tumor progression and the development of esophageal carcinoma. CBP as an effective target aiming to modulate Src signaling may be a reasonable approach to treating esophageal carcinoma.

Peer-review

This study indicates that CBP overexpression resulted in decreased growth, invasion, and migration of TE-1 cells. These findings indicate that CBP plays an important role in suppressing tumor progression. These findings are interesting, and overall the writing is good.

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ORIGINAL ARTICLE

Case Control Study

New parameter in diagnosis of acute appendicitis: Platelet distribution width

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Author contributions: Dinc B and Oskay A planned the study; Dinc B, Oskay A, Dinc SE, and Tekin S collected the data; Dinc B performed the data analysis; Dinc B and Bas B interpreted the data; Dinc B and Dinc SE wrote the paper.

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Abstract

AIM: To investigate the diagnostic accuracy of the mean platelet volume and platelet distribution width in acute appendicitis.

METHODS: This retrospective, case-controlled study compared 295 patients with acute appendicitis (Group Ⅰ), 100 patients with other intra-abdominal infections (Group Ⅱ), and 100 healthy individuals (Group Ⅲ) between January 2012 and January 2013. The age, gender, and white blood cell count, neutrophil percentage, mean platelet volume, and platelet distribution width values from blood samples were compared among the groups. Statistical analyses were performed using SPSS for Windows 21.0 software. In addition, the sensitivity, specificity, positive and negative predictive values and likelihood ratios, and diagnostic accuracy were calculated.

RESULTS: The mean ages of patients were 29.9 ± 12.0 years for Group Ⅰ, 31.5 ± 14.0 years for Group Ⅱ, and 30.4 ± 13.0 years for Group Ⅲ. Demographic features such as age and gender were not significantly different among the groups. White blood cell count, neutrophil percentage and platelet distribution width were significantly higher in Group Ⅰ compared to groups Ⅱ and Ⅲ ($P < 0.05$). Diagnostically, the sensitivity, specificity and diagnostic accuracy were 73.1%, 94.0%, and 78% for white blood cell count, 70.0%, 96.0%, and 76.0% for neutrophil percentage, 29.5%, 49.0%, and 34.0% for mean platelet volume, and 97.1%, 93.0%, and 96.0% for platelet distribution width, respectively. The highest diagnostic accuracy detected was for platelet distribution width between Group Ⅰ and Group Ⅲ ($P < 0.01$).

CONCLUSION: Platelet distribution width analysis can be used for diagnosis of acute appendicitis without requiring additional tests, thus reducing the cost and loss of time.

Key words: Appendicitis; Diagnosis; Platelet function test; Platelet distribution width

Core tip: The diagnosis of acute appendicitis can be difficult and confusing. A rapid and accurate diagnosis is important because of potential complications, therefore, new biomarkers for diagnosis are needed. This study investigated the diagnostic accuracy of indicators of platelet activation, namely mean platelet volume and platelet distribution width, in acute appendicitis patients. Results shows that platelet distribution width analysis can be used for diagnosis of acute appendicitis without requiring additional tests. Therefore, it reduces the cost and loss of time.

Dinc B, Oskay A, Dinc SE, Bas B, Tekin S. New parameter in diagnosis of acute appendicitis: Platelet distribution width. *World J Gastroenterol* 2015; 21(6): 1821-1826 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i6/1821.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i6.1821

INTRODUCTION

Acute appendicitis (AA) is among the most common urgent abdominal surgical conditions worldwide[1,2]. The people in Western society have an approximately 8% possibility of having AA during their lifetime[3]. Whereas the diagnosis of AA is usually established clinically, the symptoms and findings may not always typical, in which case the establishment of diagnosis becomes difficult[4,5]. Rapid and accurate diagnosis is important because extension of the period between the initiation of the symptoms and start of the surgical procedure increases the risk for appendiceal perforation, thereby potentially resulting in sepsis and even death[6]. In addition, the ratio of patients undergoing appendectomy with a normal histopathologic investigation result (negative appendectomy) ranges between 5% and 42%. The morbidity of these patients who are operated on despite the absence of AA is thus increased[7,8]. The rate of clinical diagnosis of AA is approximately 85%[1]. Although current advanced imaging methods such as ultrasonography, computed tomography and magnetic resonance imaging are promising, they are not adequate. Therefore, novel methods that differentiate AA from nonspecific abdominal pain and reduce the rate of negative appendectomy are needed. Such methods should be inexpensive and convenient, with results obtained in a short time.

Recent studies have investigated the diagnostic accuracy of inflammatory markers[9,10]. Mean platelet volume (MPV) and platelet distribution width (PDW) are presented in the complete blood cell count, which is routinely used in emergency departments. They are the indicators of platelet activation. The size of the platelet is correlated with the activity and the function of the platelet; larger platelets are more active than small ones. Thus, MPV may be used as a biomarker in myocardial infarction, diabetes mellitus, inflammatory disorders, sepsis-like conditions, myeloproliferative diseases, massive hemorrhage, leukemia, vasculitis and post-splenectomy conditions[11,12]. Platelet distribution width is an indicator of variation in platelet size, which can be a sign of active platelet release. Studies have demonstrated that in addition to MPV, PDW is also altered compared to healthy subjects in several conditions[13,14].

There are very few studies investigating the diagnostic accuracy of platelet function parameters in cases of AA. We present the first case-controlled study investigating the diagnostic significance of platelet parameters, including MPV and PDW, in AA.

MATERIALS AND METHODS

Participants

The records of 295 adult patients, aged between 16 and 94 years, who underwent appendectomy (Group Ⅰ), 100 patients with other intra-abdominal infections (Group Ⅱ), and 100 healthy individuals (Group Ⅲ) were retrospectively investigated between January 2012 and January 2013. The groups were compared by age, gender and routine tests of complete blood count. Group Ⅰ was comprised of patients diagnosed with AA based on the analysis of at least two samples performed by the pathologist.

Patients were excluded from the study for: < 15 years of age, having acute or chronic infectious disease, comorbid conditions (cardiac, respiratory, renal, endocrinal, and vascular disease, cancer, *etc*.), hematologic disease and blood transfusion within the last year for any reason, using ongoing medication (analgesics, oral contraceptives, antimetabolites, *etc*.), having a histopathologically normal appendix following appendectomy.

There were two control groups. The first of these, Group Ⅱ did not have AA but they had intra-abdominal infections that depended on causes such as acute cholecystitis, pelvic inflammatory disease, and mesenteric lymphadenitis. Group Ⅲ was comprised of healthy individuals, including people who came for health control and they did not have acute or chronic disease. The control groups were selected from among people of similar age and gender to AA patients. There was no history of drug use or blood transfusion in any of the patients.

Test methods

All blood samples were obtained from the venous system and stored in tubes containing EDTA and assayed automatically using internationally certified devices. The reference values were 4.5 × 10^9/μL-10.8 × 10^9/μL for white blood cell count (WBC), 40%-70% for neutrophils, 7.2-11.0 fL for MPV and 10%-18% for PDW. All results were approved by an independent biochemistry expert who was blind to the patients' histories. Extreme results were repeated again. The

Table 1 Comparison of the laboratory values between the groups

Variable	Group Ⅰ	Group Ⅱ	Group Ⅲ	*P* values			
	(*n* = 295)	(*n* = 100)	(*n* = 100)	Ⅰ *vs* Ⅱ	Ⅰ *vs* Ⅲ	Ⅱ *vs* Ⅲ	Overall
White blood cell count, $\times 10^9/\mu L$	12.9 (3.1-25.7)	11.5 (5.2-20.0)	7.5 (3.5-13.6)	0.032	< 0.001	< 0.001	< 0.001
Neutrophil, %	73.9 (18.0-93.0)	67.9 (45.0-87.9)	57.6 (21.0-76.0)	0.031	< 0.001	< 0.001	< 0.001
Mean platelet volume, fL	8.5 (6.1-14.2)	8.9 (6.0-13.0)	8.9 (6.9-14.5)	NS	0.001	NS	0.003
Platelet distribution width, %	49.0 (10.6-86.5)	40.8 (12.8-87.9)	18.4 (10.3-62.5)	< 0.001	< 0.001	< 0.001	< 0.001

Data are presented as mean (range). NS: Not significant.

Table 2 Diagnostic comparison of blood parameters

Parameter	Cutoff	AUC (95%CI)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	PLR	NLR	DA (%)
White blood cell count	$10.6 \times 10^9/\mu L$	0.87 (0.84-0.91)	73.1	94	97.1	56.0	12.1	0.3	78
Neutrophil	69.85%	0.87 (0.84-0.91)	70.0	96	97.9	54.2	23.0	0.3	76
Mean platelet volume	8.98 fL	0.62 (0.55-0.68)	29.5	49	61.1	20.1	0.5	4.5	34
Platelet distribution width	32.15%	0.95 (0.92-0.98)	97.1	93	97.4	92.1	13.8	0.0	96

AUC: Area under the receiver operating characteristic curve; DA: Diagnostic accuracy; NLR: Negative likelihood ratio; NPV: Negative predictive value; PLR: Positive likelihood ratio; PPV: Positive predictive value.

results of blood samples were approved in < 10 min.

Statistical analysis

Statistical analyses were performed using SPSS for Windows 21.0 software (IBM Corp., Armonk, NY, United States). The demographic and clinical properties of patients are expressed using mean ± SD, median (range), and percentage values. Parametric parameters were investigated with a Student's *t*-test and one-way analysis of variance, and non-parametric parameters were investigated using the Mann-Whitney *U*, χ^2 and Kruskal-Wallis tests. The association between the numeric data was compared using a correlation analysis. The parameters in the AA and control groups were described using a receiver operating characteristic (ROC) curve analysis. In addition, the sensitivity, specificity, positive and negative predictive values and likelihood ratios, and diagnostic accuracy were calculated by the area under the ROC curve. Results were evaluated within the 95%CI, and a $P < 0.05$ was considered as statistically significant.

RESULTS

The mean ages of patients were 29.9 ± 12.0 (range: 16-94 years), 31.5 ± 14.0 (range: 16-85 years) and 30.4 ± 13.0 years (range: 16-70 years) in Group Ⅰ, Group Ⅱ and Group Ⅲ, respectively. Group Ⅰ was comprised of 60.7% (179/295) men and 39.3% (116/295) women; Group Ⅱ was comprised of 50.0% (50/100) men and 50.0% (50/100) women; and Group Ⅲ was comprised of 60.0% (60/100) men and 40.0% (40/100) women. There were no demographic differences among the groups.

The comparisons of the laboratory values among the groups are given in Table 1. For all parameters, there were statistical differences between Group Ⅰ and the two control groups (*P*s < 0.05). MPV was lower in Group Ⅰ, whereas PDW, WBC (Figures 1 and 2) and neutrophil percentage were higher compared to the control groups.

The diagnostic comparisons of the blood value evaluations are given in Table 2. PDW was the most important diagnostic parameter, followed by WBC, neutrophil percentage and MPV. PDW showed high positive and low negative likelihood ratios. As a result, the diagnostic accuracy for PDW is higher than WBC and neutrophil percentage. In contrast, MPV has the lowest diagnostic accuracy, with a significant difference between Group Ⅰ and Group Ⅲ. The ROC curves for these parameters are shown in Figure 3. The high positive accuracy of PDW can also be seen here. As the MPV accuracy shows a reduction in Group Ⅰ, it is located on the negative side of the reference line as a weak positive. The ROC curve analysis is presented for MPV without correction; smaller test results used in SPSS indicate a more positive test.

DISCUSSION

AA is the most common cause of "acute abdomen" in all age groups. Although the classical symptomatology and the examination findings of AA are well known, the diagnosis remains quite difficult to make among the causes of abdominal pain[15]. It is important to make a rapid and accurate diagnosis before the complications develop[1]. As AA is an inflammatory process, many authors consider using biomarkers for diagnosis. Among these, WBC is the one most commonly used. Many studies support that WBC is the first indicator to be elevated in appendix inflammation[16]. Among patients with AA, the sensitivity and specificity of WBC is

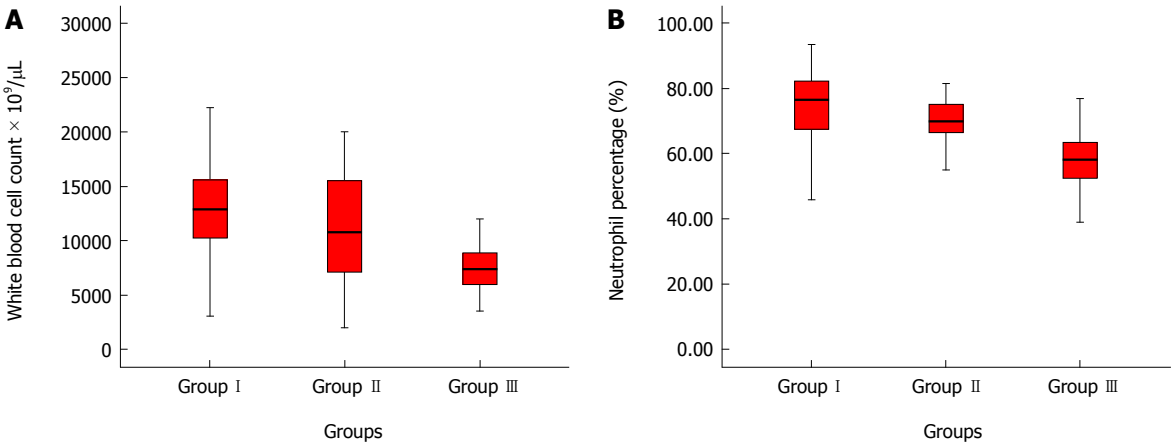

Figure 1 Blood cell counts. A: White blood cell count; B: Neutrophil percentage count variation.

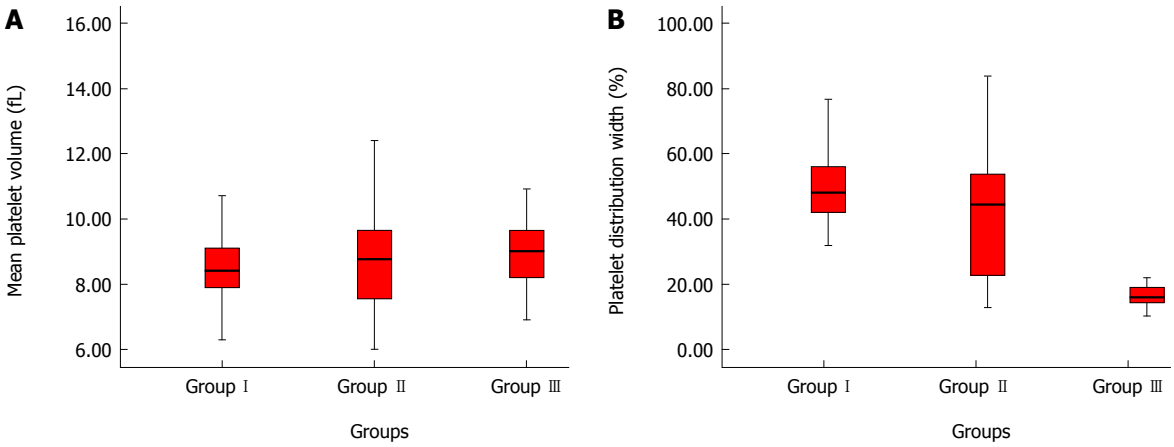

Figure 2 Platelet analyses. A: Mean platelet volume; B: Platelet distribution width variation.

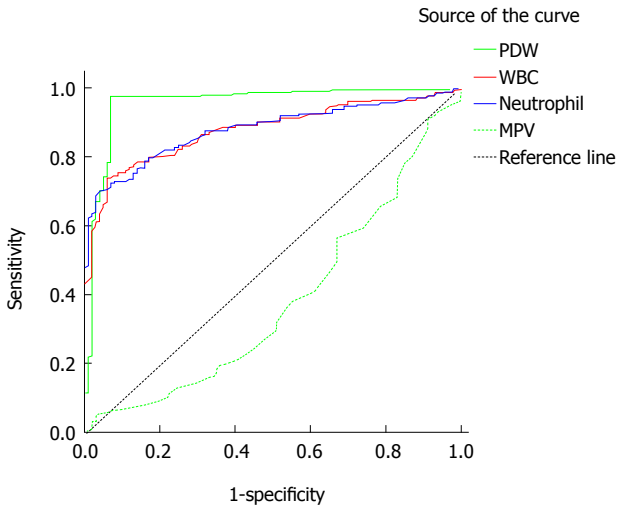

Figure 3 Receiver operating characteristic curves. MPV: Mean platelet volume; PDW: Platelet distribution width count; WBC: White blood cell count.

60%-87% and 53%-100%, respectively[15], with cut-off values of 11 × 10^9/L in the study by Bilic *et al*[9], and 10.4 × 10^3/mm^3 in the study by Narci *et al*[6], which are consistent with our findings. In addition, the 78% diagnostic accuracy enhances the significance of WBC diagnostically.

There are several studies investigating the diagnostic accuracy of neutrophils in AA. Al-Gaithy[17] reported a sensitivity and a specificity of 70.9% and 65.5%, respectively, for neutrophil detection in patients undergoing surgery for suspected appendicitis that were classified according to pathology as AA or normal. In a case-controlled study by Bilici *et al*[9] in children, the sensitivity was 77% and the specificity was 91%. In the current study, we found a sensitivity of 70% and a specificity of 96%, which is similar to the literature. The differences in ratios were potentially attributed to the variability of the individuals (adult, child, *etc.*) and the type of the designed study.

Platelet activation is related to pathophysiology of disorders with a tendency for inflammation and thrombosis. MPV, a marker of platelet activation, is being investigated for its correlation with both inflammation and thrombosis. High MPV values are associated with cardio- and cerebrovascular disorders, and low-grade inflammatory conditions prone to arterial and venous thrombosis. Low MPV values may occur in high-grade inflammatory diseases, such as active rheumatoid

arthritis or attacks of familial Mediterranean fever. These results are from anti-inflammatory treatment. As a result, a decrease in MPV occurs in acute cases, whereas an increase occurs with chronic events[18].

The proinflammatory activities of platelets are maintained by bioactive molecules stored within their alpha and dense granules[19,20]. After activation, these molecules are rapidly secreted. However, the exact organelle activity that controls the thrombocyte volume has not yet been clearly identified[19]. Danese *et al*[20] speculated that the reduced MPV could be due to the consumption or sequestration of the large activated platelets in the intestinal vasculature.

In the current trial, we detected a lower MPV in patients with AA. The cause of this remains unclear. A small number of studies failed to clearly demonstrate the correlation between MPV and AA. In the studies by Albayrak *et al*[21], Bilici *et al*[9] and Aydogan *et al*[11], MPV was significantly lower in the AA group, and with a sensitivity and specificity of 73%-84% and 54%-84%, respectively. Uyanik *et al*[15] failed to detect a correlation between AA and MPV in their study in children. However, we detected a statistical difference between the groups, and MPV was lower in the infected group. While the pathophysiology of this decrease is not known, it may be similar to the mechanism suggested by Danese *et al*[20]. The values of sensitivity and specificity of MPV in our study, 25.9% and 49.0%, respectively, are low compared to the literature. This result may stem from the emergency surgery after the clinical history and physical examination.

Both MPV and PDW are markers of platelet immaturity, and an increase in both as compared to controls suggests that young platelets are entering peripheral circulation. PDW is a function of standard deviation of log volume and is also known as the volume change coefficient. PDW is an index of thrombocyte volume heterogeneity, similar to erythrocyte distribution. The heterogeneity of thrombocyte volume occurs due to heterogenic demarcation of megakaryocytes rather than the aging of circulating thrombocytes[11].

There are a small number of studies that evaluate PDW. In a trial by Liang *et al*[22] investigating vascular dementia and Alzheimer's disease, PDW was significantly lower in the patient group compared to the control group. In contrast, Mete Ural *et al*[23] detected high PDW values in patients with recurrent miscarriages. Cetin *et al*[24] reported a higher PDW value in patients with acute ST-segment elevation myocardial infarction compared to those with stable coronary artery disease. Yang *et al*[25] detected higher PDW values in patients with severe preeclampsia relative to those with mild preeclampsia. In the literature, there exists only one study that investigates the correlation between PDW and AA. In a 202-case study by Aydogan *et al*[11], patients were divided into groups as perforated and non-perforated. In this study with no control group, PDW values were significantly higher in the perforated group. In conclusion, PDW was considered as a marker that could be used in early detection of the perforation risk in AA. In the current study, we detected high PDW values in patients with AA, for which the sensitivity, specificity and diagnostic accuracy suggest that it could help in early diagnosis of AA.

This is the first study in the literature to present the correlation between AA and PDW as a case-controlled clinical trial. Detection of higher PDW values in the AA group relative to the control groups, and the high values of sensitivity, specificity, and diagnostic accuracy detected for AA are promising for future studies in diagnosis and early prediction of potential complications. This may provide an opportunity for making a diagnosis of AA without requiring additional analysis, increased cost, or loss of time, and is practically applicable in the emergency department. Nevertheless, diagnosis of AA should always be combined with clinical, laboratory and radiologic evaluations. PDW could be an important laboratory evaluation, but should always be associated with additional signs (right lower quadrant tenderness, elevated temperature, rebound tenderness), symptoms (migration of pain, vomiting) and other laboratory tests (leukocytosis, C-reactive protein). Additional multi-center, prospective studies are needed involving a larger sample size to confirm the results of this study.

COMMENTS

Background

The diagnosis of acute appendicitis (AA) can be difficult and confusing. While the diagnosis of AA is usually established clinically, the symptoms and findings may not always be typical. Rapid and accurate diagnosis is important because of potential complications. Novel methods that differentiate AA from non-specific abdominal pain and reduce the rate of negative appendectomy are needed. Such methods should be inexpensive and convenient, with results obtained in a short time. Therefore, recent studies have been investigating the diagnostic accuracy of inflammatory markers.

Research frontiers

Mean platelet volume (MPV) and platelet distribution width (PDW) are presented in the complete blood cell count, which is routinely used in emergency departments. They are the indicators of platelet activation.

Innovations and breakthroughs

There are very few studies investigating the diagnostic accuracy of platelet function parameters in cases of AA. A diagnostic comparison of these blood values was conducted in this study. The most important parameter identified was PDW, followed by white blood cell count, neutrophil percentage and MPV. Among the laboratory evaluations for AA, PDW could be an important one, but it should always be in association with the signs (right lower quadrant tenderness, elevated temperature, rebound tenderness), symptoms (migration of pain, vomiting) and other laboratory tests (leukocytosis, C-reactive protein).

Applications

In the literature, there is no case-controlled study investigating MPV or PDW in cases with AA. This is the first study in the literature to investigate the diagnostic significance of platelet function parameters in AA.

Terminology

MPV and PDW are indicators of platelet activation and are inflammatory markers.

Peer-review

This manuscript is a well-designed, interesting, retrospective, case-controlled study. It defines a new parameter for diagnosis of AA, which is a universal problem. PDW can be an important test in the diagnosis of AA.

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ORIGINAL ARTICLE

Case Control Study

Digitally reinforced hematoxylin-eosin polarization technique in diagnosis of rectal amyloidosis

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Author contributions: Doganavsargil B and Sen S contributed equally to the conception and design of this work; Doganavsargil B and Buberal GE conducted the research and analyzed the data; Toz H collected the clinical data; Doganavsargil B, Buberal GE, and Sen S interpreted the data; Sarsik B and Sen S designed and optimized the technique used in the study; Sezak M and Sen S arranged the study and control groups; Pehlivanoglu B and Doganavsargil B drafted the manuscript; and Doganavsargil B approved the final version of the paper.
Ethics approval: Our study has been conducted according to the general approval of our hospital's ethics committee for education and investigation using the tissue slides of cases that were diagnosed in our department before August 2011. Upon the code of Turkish Ministry of Health on 19.08.2011 with the number 28030, it is confirmed that the blood, urine, tissue samples, radiology images and materials *etc*. that had been obtained with an informed consent before August 2011 are considered anonymous.
Institutional animal care and use committee: No animal-derived material was used in the study.
Conflict-of-interest: All authors have no conflict of interest related to the manuscript.
Data sharing: Technical appendix, statistical code, and dataset available from the corresponding author at bdoganavsargil@yahoo.com. Informed consent was not obtained but the presented data are anonymized and risk of identification is low, because our study simply focuses on histopathologic findings.

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Abstract

AIM: To investigate the efficacy of the digitally reinforced hematoxylin-eosin polarization (DRHEP) technique for detection of amyloidosis in rectal biopsies.

METHODS: One hundred hematoxylin-eosin (HE) stained rectal biopsies with Congo-red (CR)-positive amyloid depositions and 50 control cases with CR-negative amyloid-mimicking areas were scanned blinded to the CR results for amyloid depositions under both bright and polarized light, and digitally photographed using the DRHEP technique, to accentuate the faint birefringence observed in HE slides under polarization. The results of DRHEP and HE evaluation were statistically correlated with CR polarization results with respect to presence and localization of amyloid deposits as well as amyloid types.

RESULTS: Amyloid deposits showed yellowish-green birefringence by DRHEP, which allowed identification of amyloidosis in 41 HE-unsuspected cases (P = 0.016), 31 of which only had vascular deposits. True positivity was higher, and false negativity and positivity were lower by DRHEP, compared to evaluation by HE (69%, 31%, and 0.8% *vs* 33%, 67%, and 33%, respectively;

$P < 0.0001$). The sensitivity, specificity, accuracy, and positive and negative predictive values for DRHEP were 69%, 98%, 78.6%, 98.5%, and 61.25%, respectively. Reasons for DRHEP false negativity were presence of extensive background birefringence in 12 cases, absence of CR birefringent vessel in 3 cases, and missing of the tiny deposits in 9 cases, which could be improved by experience, especially in the latter case. No correlation was found between age, gender, sites of deposits, or amyloid types.

CONCLUSION: The DRHEP technique improves diagnostic accuracy when used as an adjunct or a prior step to CR staining, especially for cases with limited tissues for further analysis.

Key words: Amyloidosis; Congo red; Hematoxylin-eosin; Microscopy; Polarization; Rectum

Core tip: Amyloid fibrils show a faint birefringence with polarization microscopy even when they are stained with hematoxylin-eosin (HE), and this effect can be reinforced when digital images are captured. We researched the efficacy of this technique in rectal biopsies and observed that it allowed identification of unsuspected cases with HE. True positivity was higher, and false negativity and positivity were lower compared to evaluation by HE. Therefore it can be used as an adjunct or a prior step to Congo-red staining, especially for cases with limited tissues for further analysis as it is a fast and safe method.

Doganavsargil B, Buberal GE, Toz H, Sarsik B, Pehlivanoglu B, Sezak M, Sen S. Digitally reinforced hematoxylin-eosin polarization technique in diagnosis of rectal amyloidosis. *World J Gastroenterol* 2015; 21(6): 1827-1837 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i6/1827.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i6.1827

INTRODUCTION

Amyloidosis is a heterogeneous group of disorders characterized by excessive deposition of misfolded amyloid fibrils in tissues and organs. The diagnostic work-up of amyloidosis requires a multidisciplinary approach and a careful clinical evaluation; however definitive diagnosis still relies on microscopic examination of the tissues. Amyloid fibrils are seen as amorphous, eosinophilic deposits on routine hematoxylin-eosin (HE)-stained preparations, but sole HE staining is insufficient for diagnosis, as these can easily be confused with hyaline changes or sclerosis. Congo red (CR) stain and apple-green birefringence of CR-stained deposits under polarized light has long been considered as the gold standard for definitive diagnosis since it was first described by Kyle[1] in 1927.

However, despite the higher sensitivity and specificity of CR staining, false negative results can be seen due to the amount of the amyloid present in the tissue, age of amyloid deposits, thickness of the sections, fixation of the tissues, and even the staining technique itself[2]. Various methods have been investigated to increase the sensitivity of CR staining and more accurately diagnose early deposits, such as combining CR with immunohistochemistry[3] or CR fluorescence[4]. These methods are technically challenging and are not used widely for routine diagnostic purposes.

Digitally reinforced HE polarization (DRHEP) is a recently introduced technique, which was proposed as an adjunct to the diagnosis of amyloidosis in HE-stained sections in renal biopsies[5]. The technique is a combination of routine light microscopy and digital photography, and was inspired by the observation of amyloid fibrils showing a weak birefringence by polarization microscopy even in HE-stained slides[6]. This faint polarization effect is, however, more pronounced and readily identifiable in digitally captured images of the area. Therefore, utilization of digital enhancement may allow detection of birefringence that is not recognizable through the microscope objective.

Although this technique is currently being used in kidney biopsies, its utility in other specimens is not well characterized. Therefore, we investigated DRHEP in rectal biopsies, as the rectum is not only one of the most frequently biopsied sites for diagnosis of amyloidosis, but also is a target organ, as the gastrointestinal (GI) tract is one of the most commonly affected organ systems in systemic amyloidosis[7].

MATERIALS AND METHODS

Data collection, preparation of study set, and histochemical staining

A retrospective data search was conducted in the Pathology database between 2000 and 2010 using the words "rectum" and "amyloid". All cases with a clinical or histologic suspicion of amyloidosis and a Congo red stain were collected. Clinical data, including patient age and amyloid subtype, were collected from patient records within the Nephrology Department.

As reliable subtyping could not be done in all cases due to either inadequacy of tissue or technical reasons, amyloid subtypes were classified into three main groups as amyloid A (AA), amyloid light-chain (AL), or undetermined for statistical analyses.

The study set was arranged by one of the researchers who blinded other researchers to the case identities and CR results. The study group was composed of 100 rectal biopsies with CR-positive amyloid deposits, and the control group consistent of 50 CR-negative cases bearing amorphous eosinophilic

amyloid-mimicking areas.

Four-micron thickness sections were obtained from paraffin-embedded tissue blocks. HE staining was carried following standard protocols. CR staining was performed using a modified stringent, alkaline, alcoholic Putchtler's method[4].

Preparation of digital images

All HE stained sections were evaluated at low power (4×) and areas representative/suspicious for amyloid deposition were identified and photographed under both 10× and 20× magnifications. The same microscopic fields were then consecutively photographed on a dark background during polarization. CR-stained sections of the biopsies were also similarly photographed both under visible bright light and under polarization. Care was taken to capture the same or similar area that was photographed in HE images.

Digital photography was conducted according to the details of the technique described in the related reference using an Olympus BX51 polarizing microscope (Olympus Corp., Tokyo, Japan) equipped with a DP21, SAL camera[5]. In total, each case had four images: HE, DRHEP, CR, and CR with polarization (CRP).

Evaluation of HE and DRHEP images

Both HE and DRHEP images were evaluated blind to clinical data and CR staining of the biopsies, which was regarded as the gold standard. The evaluation was done independently by two researchers. In cases of disagreement, a third opinion was obtained and a consensus diagnosis was reached with two-thirds majority.

First, plain HE and DRHEP images were consecutively evaluated for the presence of suspicious amyloid depositions. The deposits showing green or yellowish-green birefringence were regarded as positive[5]. However, extensive yellowish-green birefringence was regarded as nonspecific refraction. Later, CR-stained images, which were photographed under both bright visible light and polarized light, were evaluated in a similar fashion, with investigators blinded to results of HE and DRHEP evaluations. The site of amyloid deposits, such as vascular (vessel walls), interstitial (lamina propria) or muscular (muscularis mucosae), were also recorded. Last, the results between DRHEP and CR staining were compared and discrepant cases were re-evaluated.

Statistical analysis

The frequency analyses and statistical evaluation were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, United States). The agreement between researchers was analyzed by κ analysis. The results of HE, DRHEP, and CR evaluation and their relationships with clinicopathologic features were compared by nonparametric tests (χ^2). The sensitivity, specificity, accuracy, and positive and negative predictive values for HE and DRHEP were additionally calculated.

RESULTS

Clinicopathologic features

The CR-positive study group was composed of samples from 40 women and 60 men with a mean age of 53.28 ± 14.9 years (range: 16-83 years). The mean age of the control group was 51.16 ± 15.8 years (range: 14-78 years). Patient age was not significantly different between study and control groups ($P = 0.146$). The three most common underlying etiologies were ankylosing spondylitis, familial Mediterranean fever, and rheumatoid arthritis. The subtype of amyloidosis was AA in 54 cases and AL in 9 cases. Clinical details and amyloid types of the cases are given in Table 1.

Results of HE and DRHEP evaluation

In 74.7% of the cases, the investigators agreed upon the amyloidosis status. Amyloidosis could readily be identified both in HE and DRHEP images of 28 CR-positive cases (28%) (Figure 1). Amyloid fibrils appeared as fluffy or condensed pink deposits on HE sections (Figure 1A) and showed yellowish-green birefringence on DRHEP images (Figure 1B). They stained salmon-pink/red by CR (Figure 1C) and showed apple-green birefringence by CRP (Figure 1D). However, DRHEP alone allowed identification of an additional 41 cases, which were not suspected by brightfield HE examination ($P = 0.016$), as even smaller deposits were easily identified by DRHEP because of their striking luminescence, which was comparable to CRP (Figure 2). The observed refraction pattern was specific to amyloidosis. Even though some of the foreign bodies also showed a variety of polarization patterns, they lacked the yellowish-green tint of the polarization observed in amyloid-deposited areas (Figure 2). Likewise, collagen bundles also showed a distinct white to silver polarization by DRHEP.

Sensitivity and specificity of DRHEP evaluation: The results of the HE and DRHEP image evaluation in study and control groups, as well as the sensitivity, specificity, accuracy, and positive and negative predictive values are given in Table 2.

There were only five cases that were suspected as positive by HE evaluation but were negative by DRHEP technique (Figure 3). However, the overall ratio of false-negative cases was lower by DRHEP evaluation compared to HE image evaluation alone (31 cases *vs* 67 cases, $P < 0.001$).

In addition, the ratio of false-positive cases by DRHEP was also significantly lower, as there was only one (0.8%) false-positive case among the DRHEP-positive group ($P < 0.001$) in contrast to 34 false-

Table 1 Distribution of amyloid types and clinical histories detected with digitally reinforced hematoxylin-eosin polarization and hematoxylin-eosin images among study group cases

Clinical history	Cases identified (*n*)						
	Total	By DRHEP	By DRHEP only	By both DRHEP and HE	By HE	By HE only	By neither technique
AA-type amyloidosis							
Ankylosing spondylitis	13	8	6	2	5	2	3
Familial mediterranean fever	13	8	3	5	5	0	5
Rheumatoid arthritis	10	5	4	1	2	1	4
Inflammatory bowel disease	6	5	3	2	2	0	1
Behcet's disease	3	0	0	0	0	0	3
Tuberculosis	2	2	2	0	0	0	0
Chronic obstructive lung disease	2	2	1	1	1	0	0
Still syndrome	1	1	1	0	0	0	0
Reiter syndrome	1	1	1	0	0	0	0
Psoriasis	1	0	0	0	0	0	1
TRAPS	1	1	1	0	0	0	0
Chronic infection	1	1	1	0	0	0	0
Total	54	34	23	11	15	3	17
AL-type amyloidosis							
Multiple myeloma	8	7	7	0	0	0	1
Plasma cell dyscrasia	1	1	0	1	1	0	0
Total	9	8	7	1	1	0	1
Amyloidosis-type undetermined							
Hereditary amyloidosis[1]	10	7	3	5	4	1	2
Chronic renal failure	5	3	1	2	2	0	2
Plasma cell dyscrasia	1	1	0	1	1	0	0
Malignancy	4	4	2	2	2	0	0
Unknown causes	17	12	5	6	8	1	4
Total	37	27	11	16	17	2	8
Overall total	100	69	41	28	33	5	26

[1]Transthyretin-related amyloidosis mutation was detected in one case. TRAPS: Tumor necrosis factor receptor-associated periodic syndrome; HE: Hematoxylin-eosin; DRHEP: Digitally reinforced hematoxylin-eosin polarization.

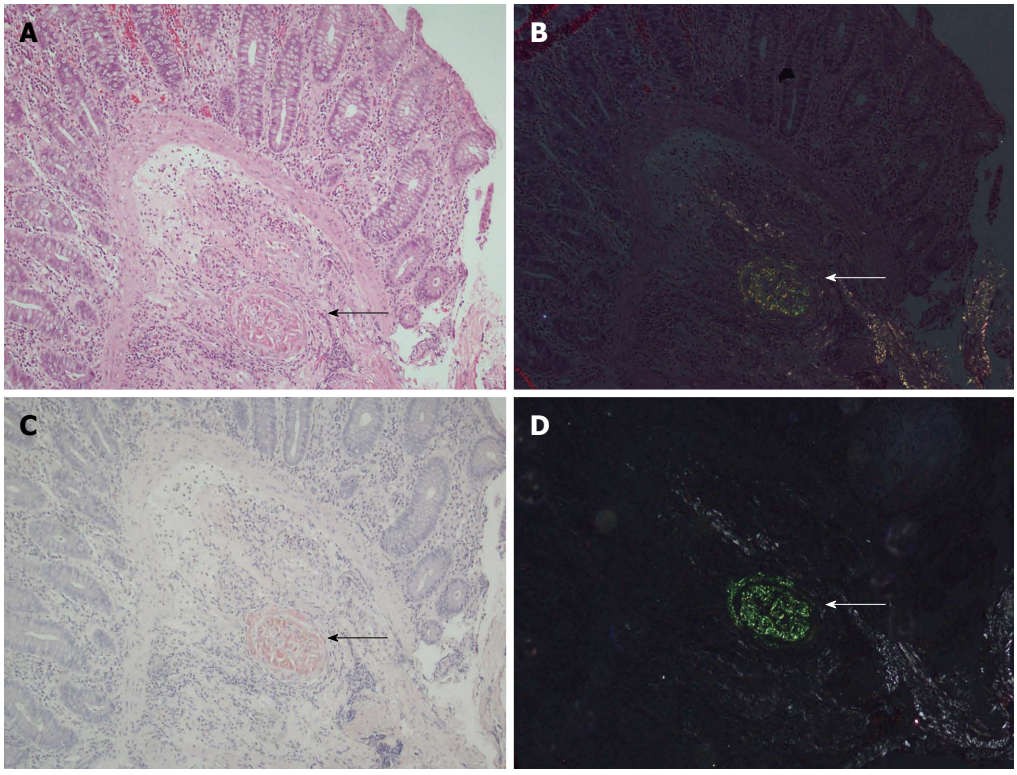

Figure 1 Easily identified amyloid deposits in a submucosal vessel wall (arrows). A: Brightfield view of hematoxylin-eosin (HE) staining (amyloid is stained pinkish-red); B: Digitally reinforced polarization view of the same HE-stained section. Note the yellowish green tint of birefringence; C: Brightfield view of Congo-red staining (deposits exhibit a salmon-pink appearance); D: Polarization view of the same Congo-red-stained section. Note the apple green tint of polarization is more pronounced than in the digitally reinforced HE polarization image (arrow); 10× magnification.

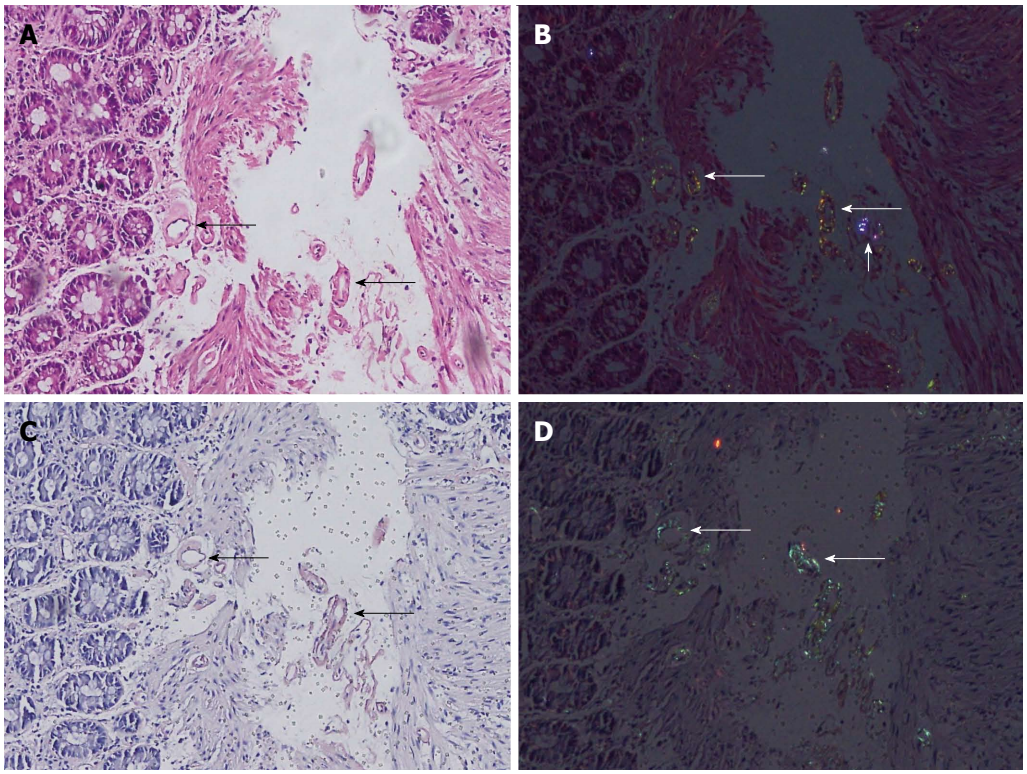

Figure 2 Tiny amyloid deposits in vessel walls, which are not striking at first sight (long arrows). A: Brightfield view of hematoxylin-eosin (HE) staining; B: Digitally reinforced polarization view of the same HE-stained section. Note the striking yellowish green tint of birefringence of amyloid in small vessel walls (long arrows) and variant colored nonspecific refraction of a foreign body (short arrow); C: Brightfield view of Congo-red staining (note the salmon pink color); D: Polarization view of the same Congo-red-stained section with apple green birefringence of deposits (arrows); 20× magnification.

Table 2 Comparison of hematoxylin-eosin and digitally reinforced hematoxylin-eosin polarization evaluated by Congo-red stain *n* (%)

Variable	DRHEP	HE
Congo red positive (n = 100)		
True positive	69 (69)	33 (33)
False negative	31 (31)	67 (67)
Congo red negative (n = 50)		
True negative	49 (98)	16 (20)
False positive	1 (4)	34 (80)
Sensitivity	69%	34%
Specificity	98%	32%
Accuracy	78.6%	33.3%
Positive predictive value	98.5	50.0
Negative predictive value	61.3	19.5

HE: Hematoxylin-eosin; DRHEP: Digitally reinforced hematoxylin-eosin polarization.

positive cases by HE evaluation only. Whether this false-positive case had an undetermined birefringent foreign body or a small amyloid deposition, which could be lost on consecutive sections, remains unclear. Overall the sensitivity, specificity, accuracy, and positive and negative predictive values for DRHEP technique were calculated as 69%, 98%, 78.6%, 98.5% and 61.25% while they were 34%, 32%, 33.3%, 50%, and 19.5% for HE evaluation respectively (Table 2).

Effect of amyloid type and site of deposition: The sites of the amyloid depositions observed by DRHEP and the effect of the DRHEP technique in identification are summarized in Table 3. It was easier to identify or suspect mixed vascular, interstitial, and muscular deposits than only vascular ones both with HE and DRHEP. Although no statistically significant correlation was found between the underlying etiologies or amyloid subtypes, DRHEP identified more cases regardless of site of distribution (Table 3).

Identification by DRHEP was not affected by the type of amyloid deposition (Table 1). However, it is noteworthy that some of the cases, such as myeloma and tuberculosis, were only evaluated as positive by DRHEP while they were not suspected by HE examination.

Although no significant correlation was found between age, gender, the sites of amyloid deposition, and amyloid type, the ratio of false positivity was higher in patients in the fifth decade (n = 15 cases; P = 0.049) if evaluated by HE alone in contrast to only one age-matched false-positive case (0.8%) in the DRHEP group.

Comparative evaluation of false-negative DRHEP cases with CRP

Thirty-one false-negative cases by DRHEP were re-evaluated in comparison with the CRP images. Nine

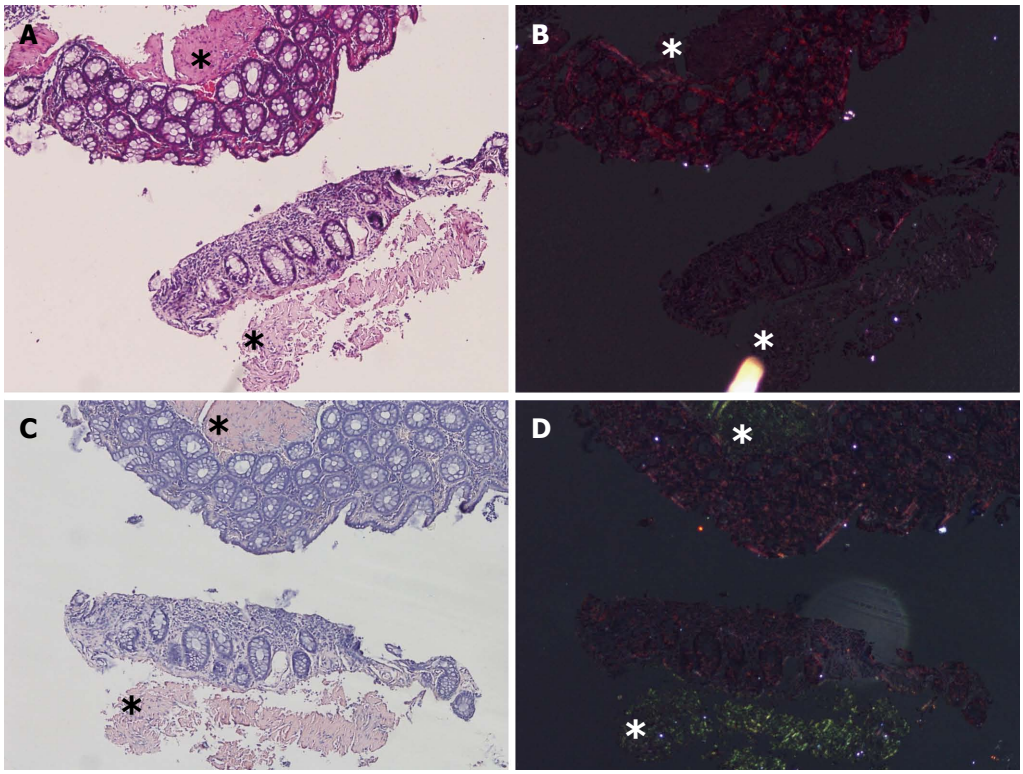

Figure 3 Amyloid suspicious deposits in muscularis mucosa and submucosa (stars). A: Brightfield view of hematoxylin-eosin (HE) staining; B: Digitally reinforced polarization view of the same HE-stained section. Note the nonspecific refractions throughout the tissue and the aberrant artificial refraction of a foreign body on the slide just below the (stars) in A and B. No convincing birefringence for amyloidosis is observed in the suspected area (stars) by HE; C: Brightfield view of Congo-red staining with salmon pink deposits (stars); D: Polarization view of the same Congo-red-stained section with apple green birefringence of deposits (stars); 10× magnification.

Table 3 Distribution of hematoxylin-eosin and digitally reinforced hematoxylin-eosin polarization evaluation results for identifying the site of deposits confirmed by Congo-red polarization, *n*

Site of deposition	Confirmed by CRP	Identified by DRHEP						Identified by HE					
		Total	*P* value	HE-/ DRHEP+	*P* value	HE+/ DRHEP+	*P* value	Total	*P* value	HE+/ DRHEP-	*P* value	HE-/ DRHEP-	*P* value
Vascular deposits only	74	48	0.24	31	0.982	17	0.147	21	0.381	4	0.823	22	0.407
Mixed deposits	26	21	0.24	10	0.982	11	0.147	12	0.381	1	0.823	4	0.407
Vascular, muscular	13	9	0.24	5	0.982	4	0.147	5	0.381	1	0.823	3	0.407
Vascular, muscular, interstitial	7	6	0.24	2	0.982	4	0.147	4	0.381	0	0.823	1	0.407
Vascular, interstitial	6	6	0.24	3	0.982	3	0.147	3	0.381	0	0.823	0	0.407
Total number of cases	100	69	-	41	-	28	-	33	-	5	-	26	-

HE: Hematoxylin-eosin; DRHEP: Digitally reinforced hematoxylin-eosin polarization; CRP: Congo-red polarization.

(29%) cases showed positive birefringent deposits that were more easily identified on CRP images. In some of those cases, background refraction obscured the tiny deposits (Figure 4).

Apart from the nine missed cases, the most common cause of false negativity was the presence of extensive yellowish-green birefringence (38.7%), which was interpreted as nonspecific refraction. Loss of the CR positive birefringent vessel (foci) (9.7%) on the DRHEP image was the other common cause for false negativity.

Similarly, the positive foci on DRHEP images were more prominent in comparison to the two false-negative cases by CR staining. Table 4 summarizes the causes for the discrepancy and their relationship with amyloid subtypes and clinical etiologies.

In five of the false-negative cases, the deposits were inexplicably non-birefringent in DRHEP images, though they were all positive by CR (Figure 3).

DISCUSSION

Amyloidosis is still an etiopathogenetically mysterious disease, since its first description by Nicolaus Fontanus in 1639[1]. To date, 30 different human fibril proteins have been identified in localized or

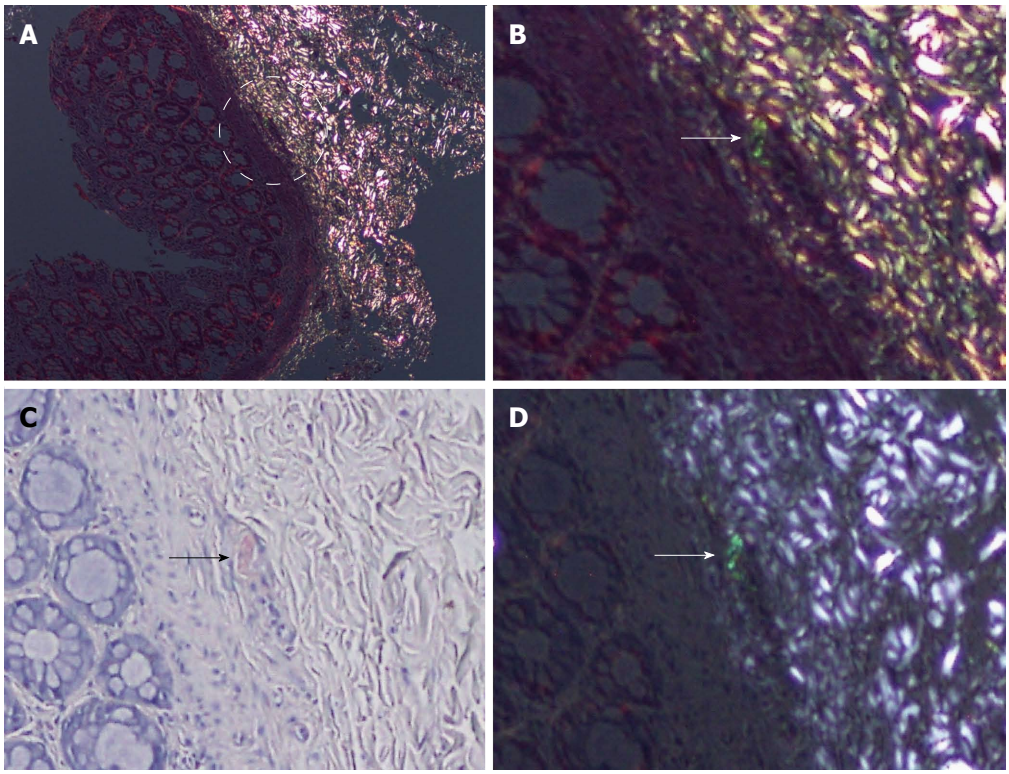

Figure 4 False negative case with extensive refraction and tiny deposits in digitally reinforced polarization view. A: No arresting positivity was observed in initial evaluation. Note the black-appearing area in the center of the circle [hematoxylin-eosin (HE), under polarization, 10×]; B: High-power digitally reinforced polarization view of the area in circle in panel A. The yellowish green birefringence in a small vessel (arrow) is distinguished after careful examination in comparison with the Congo-red image (HE, under polarization, 40×); C: Congo-red stain positivity in the same vessel (arrow) (bright field, 40×); D: Apple green birefringence of Congo-red polarization of the same vessel (arrow) (under polarization, 40×).

Table 4 Distribution of causes of false negativity by digitally reinforced hematoxylin-eosin polarization and clinical histories

Cause of false negativity	Total number of cases, *n* (%)	Clinical history	*n*	Amyloid type
Extensive refraction	12 (38.7)	Ankylosing spondylitis	2	AA
		Familial Mediterranean fever	1	AA
		Rheumatoid arthritis	1	AA
		Behcet's disease	1	AA
		Psoriasis	1	AA
		Multiple myeloma	1	AL
		Chronic renal failure	1	Undetermined
		Hereditary	1	Undetermined
		Unknown causes	3	Undetermined
Tiny deposit ("Missed cases")	9 (29.0)	Ankylosing spondylitis	2	AA
		Familial Mediterranean fever	1	AA
		Rheumatoid arthritis	2	AA
		Inflammatory bowel disease	1	AA
		Behcet's disease	1	AA
		Hereditary	1	Undetermined
		Unknown causes	1	Undetermined
Absence of the positive vessel	3 (9.7)	Familial Mediterranean fever	1	AA
		Behcet's disease	1	AA
		Ankylosing spondylitis	1	AA
Perivascular illuminance	2 (6.5)	Familial Mediterranean fever	1	AA
		Rheumatoid arthritis	1	AA
Reason unidentified (Real negative? cases)	5 (16.1)	Familial Mediterranean fever	1	AA
		Rheumatoid arthritis	1	AA
		Chronic renal failure	1	Undetermined
		Unknown causes	1	Undetermined
		Hereditary	1	ATTR
Total number of cases	31 (100.0)		31	

AA: Amyloid A; AL: Amyloid light-chain; ATTR: Transthyretin-related amyloidosis

systemic forms of the disease[8]. Although amyloid fibril formation results from protein misfolding due to several factors such as mutations or proteolysis, it is not clear how these different proteins, with different amino acid sequences, three-dimensional structures, and biologic functions, convert to a fibrillary form, which has a considerably uniform morphology. Even though any protein deposits that stain with CR and exhibit green birefringence by polarization microscopy are considered as amyloid[9], we do not know how these different proteins with different physical orientations show similar birefringence under polarized light. Regardless of the mechanisms of this polarization, our results show that amyloid fibrils have a polarization property[6] and retain this polarization effect even after staining with HE, and can be detected easily if certain conditions are met. Within the scope of this study, this polarization effect was enhanced *via* a digital camera attached to the polarization microscope, which was set up according to the method described in the original literature[5].

Using the method of Sen *et al*[5], amyloid fibrils show a constant yellowish-green tint under polarization on HE-stained sections, and can be detected in rectal biopsies. Although the color was different than observed in polarization of CR-stained sections, it was easily identifiable in most of the cases. The physical basis of the constant yellowish-green color observed during HE polarization and the effect of digital enhancement on this polarization is beyond the scope of this article given the polarization mechanisms observed in CR-stained sections are not fully uncovered yet[10]. Of note, Howie *et al*[11] pointed to different colors diverging than the classically defined apple-green tint appearing during CR polarization. Therefore, the color we observed may vary in different camera settings. The physical basis of colors seen DRHEP requires further studies dedicated to this issue. Regardless of the mechanisms, this polarization enabled visualization of even the very tiny deposits and facilitated identification of amyloidosis with 69% sensitivity. The specificity of the technique was as high as 98%, and the observed yellowish-green color with polarization was almost unique to amyloidosis, which was comparable to CRP, as false positivity was not observed apart from one case with a tiny vascular and suspicious birefringence.

We must highlight that the aim of the study is neither asserting DRHEP as a substitute for CR nor for recommending rectal biopsies for scanning of amyloidosis in rectal biopsies. CR has long been regarded as the gold standard for diagnosis of amyloidosis with higher sensitivity and specificity ratios, and abdominal fat-pad aspiration biopsies have been favored for scanning of amyloidosis since the 1970s as a safe, simple, and fast method[12,13]. However, rectal biopsies are also still in use for diagnostic purposes in our routine practice[14]. Rectal biopsies are challenging because of the more collagenous and muscular tissue structure compared to kidney and subcutaneous fat tissue samples. The reported sensitivity of rectal biopsies in detection of amyloidosis varies within the literature ranging from 69% to 97%, and is largely affected by tissue sampling[15].

Identification of amyloidosis in rectum can also be valuable for prediction of renal involvement, as almost ninety percent of GI amyloidosis also have renal amyloid deposits[7,16]. Thus, GI biopsies can also be used with comparable convenience and sensitivity[17], and efforts to increase the diagnostic sensitivity of rectal biopsies is important. In this regard, DRHEP is valuable for distinguishing tiny amyloid deposits from sclerosis, hyalinization, or other red-pink colored structures, as they do not show the yellowish-green polarization observed in amyloidosis. Significant false positivity by HE evaluation in older patients (*i.e.*, after the fifth decade) in our study group was meaningful in this respect, as it likely reflects hypertensive vascular changes that might have a similar morphologic appearance with amyloidosis and cause diagnostic difficulties. The higher specificity of DRHEP (96%) is therefore valuable.

The accurate diagnosis of amyloidosis in rectal biopsies is not only important for scanning purposes in clinically suspected cases, but also crucial in distinguishing it from its histologic mimics observed in the GI tract, such as collagenous colitis[18], ischemic bowel disease[19], and elastosis or elastofibromatous changes[20], which may cause important diagnostic challenges in inadequate biopsies. Moreover, many of the pathologies seen in the GI tract increase the risk of amyloidosis, such as inflammatory bowel disease[21,22], intestinal Behcet's[23], or chronic infections as tuberculosis[24]. Therefore a concurrent case of amyloidosis can easily be overlooked, especially in clinically unsuspected cases. However, all these diagnostic and differential diagnostic considerations indeed rely on histologic suspicion of amyloidosis in HE-stained sections, particularly when the case is not suspected clinically. As we observed in our study design, blinded review of the cases caused false negativity in 67% of the cases by sole HE evaluation; the lower sensitivity and specificity ratios might result in either missing the case totally or application of excessive CR stains to all suspected tissue blocks. In this respect, we propose that DRHEP can be used as an adjunct to both HE and CR to increase their efficacy in detecting amyloidosis. It may be of help in selecting tissue blocks for further analysis and reduce time and extra costs.

Once the polarization microscope and attached digital camera are set up, this method is easy for scanning virtually all suspected cases during routine sign-out. In fact, in three of the study group cases, clinically unsuspected amyloidosis was initially dia-

gnosed from rectal biopsies taken for other reasons, and DRHEP helped to pick up the cases as an initial step of evaluation. It was also valuable as an adjunct to CR stain in three more cases where the amyloid deposit was more extensive in HE-stained sections, and where the amyloid-bearing vessel was located at the edge of the biopsy and missed during serial cuts for CR staining. Multiple serial sections are needed to confirm the diagnosis by positivity in another vessel. Thus, we may benefit from this technique in routine practice, and with considerable safety, as the false-positivity ratio is significantly lower, comparable to CR staining. However, DRHEP should not be used as a replacement for routine CR staining, as there are still false-negative cases despite a significantly lower false-negativity ratio than with HE alone. The most common cause of false negativity was the presence of an extensive yellowish-green background birefringence (38.7%), which was suspicious for a nonspecific refraction. Although different from the silvery-white refraction of collagenous tissue, the visual complexity carried the risk of hiding true-positive tiny deposits, as we experienced in two of the cases. Loss of the CR-positive vessel in the HE section and nonspecific perivascular illumination were the other causes of false negativity. The importance of the perivascular birefringence is not known yet; whether it reflects a true deposit too tiny to be discriminated by light microscopy could not be ruled out within the scope of this study. However, this finding is worth mentioning as some of the sections also showed a sort of refraction in CRP images.

For the missed true-positive cases, we acknowledge that the recognition of the yellowish-green coloring upon polarization may require some experience and adjustment to the technique. After identifying the positive areas on CR, it was easier to comment on the observed refraction for discrepant cases. This troubleshooting or improved evaluation effect had a positive impact, particularly on the identification of tiny deposits. Thus, we believe that experience and increased practice may help to overcome some of these discrepancies.

The greater number of etiologically unknown cases, or cases in which the amyloid typing could not be further evaluated (other than AA or AL), is the major shortcoming of this study. Therefore further research is needed to uncover the effect of underlying etiology and amyloid types in relation to their visualization by DRHEP technique.

Today, many laboratories have adequate equipment (polarization attachment, polarization filter, and digital camera) for use with the DRHEP technique. Once the camera is attached and digital photography settings are made, it can be easily applied during routine biopsy evaluation with convincing safety. Laboratories can do their own optimizations. DRHEP may also be of help in reducing unnecessary CR staining in different tissue blocks of an individual case, which will save a considerable amount time, resources, and even tissue, in daily routine practice. It might even be valuable for the initial screening of cases of clinically unsuspected amyloidosis as a prior step or adjunct to CR evaluation[10,15].

The utility of a grading system as previously described in renal biopsies[25] and whether it can be applied to rectal biopsies should also be further evaluated. A standardized diagnostic approach and reporting, including the histologic structure of the deposits, should also be considered for all cases[26].

As mentioned above, the physical basis of this polarization, the effect of the CR-staining technique[27], or usage of a digital camera deserves further investigation. Dedicated research is also needed to reveal the relationship between the observed polarizations and the protein infrastructure of the deposits. The efficacy of the technique and its reproducibility in other tissues should also be investigated.

The data presented here will hopefully increase awareness about the DRHEP method and its efficacy as an adjunct to diagnosis in colorectal biopsies. To our knowledge, this is the first study dedicated to HE polarization in colorectal tissues.

COMMENTS

Background

Amyloidosis is a disparate set of disorders characterized by excessive deposition of amyloid fibrils in tissues and organs, rather than a single disease-entity with known causes and consequences. It may cause a wide range of clinical symptoms related with the involved target-organ dysfunction. Amyloid fibrils are composed of 30 different proteins, but have a considerably uniform histologic morphology. The gold standard for diagnosis is demonstration of amyloid fibril deposits in tissues, which appear as homogenous deposits that stain salmon pink with Congo red, and exhibit an apple-green birefringence when scanned with a polarization microscope. However, tissue diagnosis may be troublesome, especially in small biopsies when there is a paucity of amyloid deposits or when the tissue is too small for further staining.

Research frontiers

In a previous study on renal amyloidosis, amyloid fibrils were shown to have a polarization property when scanned with a polarized microscope, even when they were stained with a standard hematoxylin-eosin stain that is used in conventional microscopic examination of tissues. However, this effect is more readily identifiable in digitally captured images as an unrevealed effect of the camera systems attached to the microscope. The utility of this technique, known as digitally reinforced hematoxylin-eosin polarization (DRHEP), has not been researched in other tissues. This is the first study to evaluate DRHEP for detecting amyloidosis in rectal biopsies.

Innovations and breakthroughs

The rectum is not only one of the most frequently biopsied sites for diagnosis of amyloidosis, but also is a target organ, as the gastrointestinal tract is one of the most commonly affected organ systems in systemic amyloidosis. Moreover, amyloidosis may complicate many primary pathologies of the gastrointestinal tract or can mimic them, requiring additional workups for either diagnosis of amyloidosis or for ruling it out. However, the rectal biopsies are often too small for further analysis. The results of the present study show that the striking yellowish-green birefringence of amyloid fibrils with DRHEP enables identification of even very tiny deposits, and that the true-positivity ratio of DRHEP is higher, while the false-negativity ratio is lower, than examination

by hematoxylin-eosin without applying polarization. The extremely low false positivity is comparable to Congo-red staining results, rendering DRHEP a sensitive and specific method for diagnosis of amyloidosis, improving diagnostic accuracy. It can be used as an adjunct or a prior step to Congo-red staining, especially for cases with limited tissues for further analysis.

Applications

Today, many laboratories have adequate equipment (polarization attachment, polarization filter, and digital camera) for use with the DRHEP technique. Thus, it is a relatively easy method to scan virtually all suspected cases during routine sign-out.

Terminology

Hematoxylin-eosin is the conventional stain used in pathology to evaluate tissues. Amyloid fibrils are seen as pinkish-red deposits under brightfield microscopy that cannot be differentiated from fibrosis, muscle tissue, or hyalinosis, with its sole appearance by this stain. Congo red, which is an aniline dye also used in the textile industry, is used for demonstrating amyloidosis in tissues. Congo-red staining is regarded as the gold standard for diagnosis of amyloidosis, and amyloid fibrils appear salmon pink with this stain. Polarized light microscopy is used to reveal polarized (light waves vibrating in only one plane) properties of tissues. Amyloid fibers show apple-green birefringence under polarized light. DRHEP is a novel technique to detect polarization of amyloid fibers under a polarization microscope without staining with Congo-red dye. The polarization effect of amyloid fibrils that are too faint to be seen with standard hematoxylin-eosin stain is more readily identifiable when digital images are captured, and amyloid fibers show yellowish-green birefringence in these images.

Peer-review

This manuscript is an interesting paper on a niche topic and is well documented.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Fibroblast growth factor receptor 4 protein expression and clinicopathological features in gastric cancer

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Abstract

AIM: To investigate fibroblast growth factor receptor 4 (FGFR4) protein expression in Chinese patients with resectable gastric cancer (GC) and the association with clinicopathological characteristics and survival.

METHODS: One hundred and seventy-five GC patients who underwent curative surgical procedures were enrolled in this study. The protein expression of FGFR4 in formalin-fixed, paraffin-embedded (FFPE) GC tissues was determined by immunohistochemical (IHC) analysis. Patient clinicopathological data and survival information were also collected and χ^2 statistical analysis was performed to analyze FGFR4 protein expression in the subgroups with differing clinicopathological characteristics including; gender, age, tumor location, differentiation, tumor-node-metastasis stage, macroscopic type, depth of invasion, lymph node metastases, distant metastasis, neural invasion and vascular invasion. Furthermore, some common molecular markers of GC in our cancer center, including p53, p27, topoisomerase Ⅱα (Topo Ⅱα) were also determined by IHC and their association with FGFR4 protein expression evaluated. The probability of survival for different subgroups with different clinicopathological characteristics was calculated using the Kaplan-Meier method and survival curves plotted using the log rank test.

RESULTS: Seventy seven cases (44%) were found to have high expression of FGFR4 protein. Significantly different FGFR4 expression was observed between gastric cancers with differing expression of Topo Ⅱα (log rank χ^2 = 9.4760, P = 0.0236). No significant differences were observed between subgroups defined by any of the other clinicopathological characteristics. The median survival time of the FGFR4 high expression (77 cases) and low expression groups (98 cases) was 27 mo and 39 mo, respectively. The five-year survival rates and median survival times of gastric cancers with high FGFR4 expression were worse than those with low expression (30.8% *vs* 39.2%, 27 mo *vs* 39 mo), respectively, however, no significant difference was observed in survival time (log rank χ^2 = 1.0477, P = 0.3060). Survival analysis revealed that high expression of FGFR4 was a predictor of poor outcome in GC patients if the tumor was small (less than or equal to 3 cm in size) (log rank χ^2 = 5.5033, P = 0.0190), well dif-

ferentiated (log rank χ^2 = 7.9757, P = 0.0047), and of T1 or T2 stage invasion depth (log rank χ^2 = 4.8827, P = 0.0271).

CONCLUSION: Our results suggest that high tumor expression of FGFR4 protein is not an independent risk factor for GC cancer initiation, but is a useful prognostic marker for GC patients when the tumor is relatively small, well differentiated, or in the early stages of invasion.

Key words: Gastric cancer; Fibroblast growth factor receptor 4; Protein expression; Clinicopathological characteristics; Prognosis

Core tip: This study investigated the possible contributions of fibroblast growth factor receptor 4 (FGFR4) protein expression as a risk factor for gastric cancer (GC), and the associations between protein expression and clinicopathological parameters. The results suggested that FGFR4 protein expression may correlate with the expression of Topo Ⅱα. Furthermore, we demonstrated that FGFR4 protein expression is not a risk factor for GC initiation, but may be a useful prognostic marker for GC patients with tumors which are relatively small, well differentiated, or in the early stages of invasion.

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INTRODUCTION

The overall survival of patients with gastric cancer (GC) continues to improve due to the introduction of multidisciplinary treatment approaches and the identification of novel targeted agents; however, GC remains the fourth most commonly diagnosed cancer and the second leading cause of cancer-related deaths worldwide[1,2]. It remains of great clinical importance to identify new biomarkers for early diagnosis, targeted treatment and prognostic evaluation in gastric cancer.

The human fibroblast growth factor receptor (FGFR) 4 protein belongs to the FGFR family of receptor tyrosine kinases, which are involved in the regulation of diverse cellular processes including cell growth, differentiation, survival, and migration[3]. Targeting of such receptors with novel drugs is a proven therapeutic strategy, as exemplified by the clinical success of trastuzumab in treating patients with HER2 amplified breast cancer[4]. The upregulation of FGFR4 protein expression occurs in prostate[5], breast[6], pancreatic[7], renal[8] and ovarian cancers[9], and has been associated with resistance to chemotherapy in breast cancer[10]. A growing body of research indicates that inhibition of the FGF pathway may present an effective therapeutic option for cancer. Moreover, activation of the FGFR pathway may, in some cases, provide a mechanism of resistance against current targeted and antiangiogenic drugs[11].

A recent report showed that high expression of FGFR4 protein accelerated the progression of advanced GC and might be associated with poor disease prognosis in GC patients[12]. To our knowledge, this is the only report on the association between FGFR4 protein expression and GC progression in Chinese patients, and therefore requires further confirmation. Importantly, to date no studies have been conducted on the correlation between FGFR4 protein expression and the risk of GC. In this study, we investigated the expression of FGFR4 protein in the context of clinicopathological features and patient prognosis, using an expanded population of 175 Chinese patients with resectable GC.

MATERIALS AND METHODS

Patients

A retrospective cohort study was conducted and included 175 GC patients who underwent curative surgery at Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, from August 2006 to March 2009. We reviewed the medical charts and pathological records for clinicopathological parameters such as age, gender, histological subtype and pathological stage. Formalin-fixed, paraffin-embedded samples of tumors were evaluated for FGFR4 protein using immunohistochemical (IHC) analysis. None of the patients had undergone preoperative chemotherapy, radiation or targeted therapy. The study included 50 women and 125 men aged 28 to 85 years. The median age was 62 years. The tumor sample characteristics of all 175 cases are shown in Table 1. Of all the tumors examined, 32 (18.28%) were located in the cardiac region, 71 (40.58%) in the body, and 72 (41.14%) in the pylorus. 76 (43.43%) cases were poorly differentiated (grades Ⅰ and Ⅱ), and 99 (56.57%) cases were well differentiated (grades Ⅲ and Ⅳ). Tumor-node-metastasis (TNM) classification revealed that 37 cases were stage Ⅰ (21.14%), 45 were stage Ⅱ (25.71%), 69 were stage Ⅲ (39.43%) and 24 were stage Ⅳ (13.71%). Clinical stage was determined according to the Union for International Cancer Control TNM staging system, and tumor grade was based on the World Health Organization classification. Postoperative follow-up ended in March, 2014.

Immunohistochemical staining

Tissue sections of paraffin-embedded formalin-fixed tissue blocks were deparaffinized with xylene for 5

Table 1 Correlation of fibroblast growth factor receptor 4 expression with clinicopathological characteristics *n* (%)

Clinicopathological characteristics	*n*	FGFR4 positive	FGFR4 negative	χ^2	*P* value
	175	77 (44.00)	98 (56.00)		
Gender				0.1140	0.8663
Male	125	56 (44.8)	69 (55.20)		
Female	50	21 (42.00)	29 (58.00)		
Age (yr)				0.9211	1.0000
< 60	72	32 (44.44)	40 (55.56)		
⩾ 60	103	45 (43.69)	58 (56.31)		
Tumor size				0.1400	0.1518
⩽ 3 cm	60	31 (51.67)	29 (48.33)		
> 3 cm	115	46 (40.00)	69 (60.00)		
Tumor location				1.3942	0.4980
U	32	17 (53.13)	15 (46.88)		
M	71	29 (40.85)	42 (59.15)		
L	72	31 (43.06)	41 (56.94)		
Tumor differentiation				0.2191	0.1129
Poor	76	29 (38.16)	47 (61.84)		
Well	99	48 (48.48)	51 (51.52)		
Macroscopic type					
EGC	21	8 (38.10)	13 (61.90)	9.3842	0.0522
Ⅰ	9	3 (33.33)	6 (66.67)		
Ⅱ	5	3 (60.00)	2 (40.00)		
Ⅲ	127	62 (48.82)	65 (51.18)		
Ⅳ	13	1 (7.69)	12 (92.31)		
TNM stages				0.3499	0.9504
Ⅰ	37	15 (40.54)	22 (59.46)		
Ⅱ	45	21 (46.67)	24 (53.33)		
Ⅲ	69	30 (43.48)	39 (56.52)		
Ⅳ	24	11 (45.83)	13 (54.17)		
T				0.9523	0.8128
1	21	8 (38.10)	13 (61.90)		
2	29	14 (48.28)	15 (51.72)		
3	64	30 (46.88)	34 (53.13)		
4	61	25 (40.98)	36 (59.02)		
N				1.8160	0.6115
0	55	27 (49.09)	28 (50.91)		
1	32	11 (34.38)	21 (65.63)		
2	24	11 (45.83)	13 (54.17)		
3	64	28 (43.75)	36 (56.25)		
M				0.0379	1.0000
0	151	66 (43.71)	85 (56.29)		
1	24	11 (45.83)	13 (54.17)		
Neural invasion				0.7576	0.5146
Yes	25	9 (36.00)	16 (64.00)		
No	150	68 (45.33)	82 (54.67)		
Vascular invasion				0.4473	0.2356
Yes	35	13 (37.14)	22 (62.86)		
No	140	64 (45.71)	76 (54.29)		
P53				3.0941	0.3773
0	72	37 (51.39)	35 (48.61)		
1	43	16 (37.21)	27 (62.79)		
2	20	7 (35.00)	13 (65.00)		
3	40	17 (42.50)	23 (57.50)		
P27				0.9924	0.8031
0	87	37 (42.53)	50 (57.47)		
1	72	34 (47.22)	38 (52.78)		
2	12	4 (33.33)	8 (66.67)		
3	4	2 (50.00)	2 (50.00)		
Topo Ⅱα				9.4760	0.0236
0	83	29 (34.94)	54 (65.06)		
1	66	31 (46.97)	35 (53.03)		
2	25	14 (60.87)	9 (39.13)		
3	3	3 (100)	0 (0)		

FGFR4: Fibroblast growth factor receptor 4; TNM: Tumor-node-metastasis.

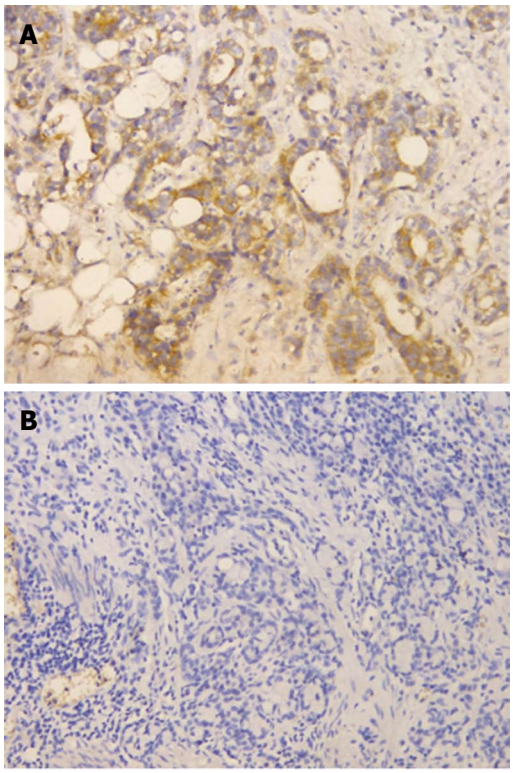

Figure 1 Immunohistochemical analysis of fibroblast growth factor receptor 4 protein expression (× 200). A: High expression; B: Low expression.

min, followed by two washes with 100% ethanol for 10 min each. The slides were then incubated in 95% ethanol for 10 min and washed twice in dH_2O for 5 min. Antigen retrieval was performed by placing the slides in 10 mmol/L citrate buffer (pH 6.0) and microwave treatment for 15 min. Tissue sections were cooled to room temperature (RT), and washed with phosphate-buffered saline (PBS) and distilled water. IHC was carried out on 4-μm sections using specific antibodies against FGFR4 (sc-124, Santa Cruz), p53 (sc-126, Santa Cruz), p27 (sc-393380, Santa Cruz), and Topo Ⅱα (sc-65743, Santa Cruz). IHC samples were examined by two pathologists who were experienced in gastrointestinal cancers and unaware of the clinical information. Immunostains were standardized using appropriate positive and negative controls for each antibody.

The FGFR4 was evaluated according to both the signal intensity and the percentage of stained cells. The signal intensity was scored as negative (0), weak (1), moderate (2) or strong (3). When the percentage of FGFR4 immune-positive tumor cells was considered, a score of 1 was given when < 10% of cells were positive; 2 when 10%-50% of cells were positive and 3 when > 50% of cells were positive. Both scores were multiplied and the resulting score was used to categorize FGFR4 expression as low expression (< 3) or high expression (> 3).

The expression of p53, p27 and Topo Ⅱα were

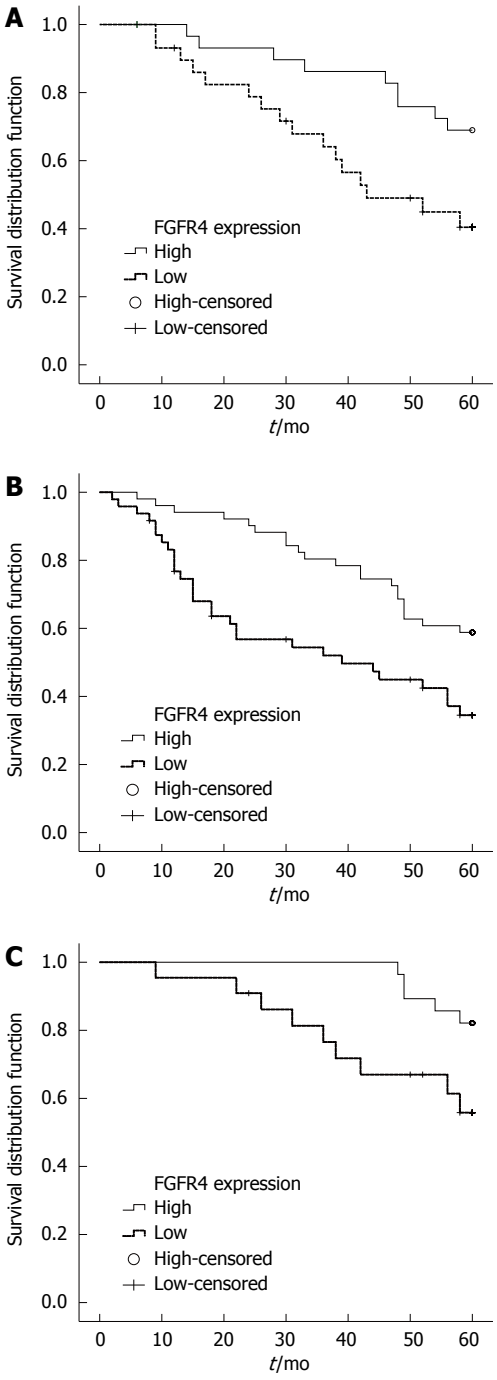

Figure 2 Significant difference was found between patients with high and low fibroblast growth factor receptor 4 protein expression after stratified Kaplan-Meier survival analysis. A: Patients with tumor size ⩽ 3 cm; B: Patients with well-differentiated gastric cancer; C: Patients with gastric cancer classified as stage T1 or T2.

assessed by determining the number of positively stained nuclei, with less than 10% of stained cells indicating a negative result. A score of 1 was given when 10%-30% of the cells stained positively. Scores of 2 or 3 were given when 30%-50% or > 50% of the cells stained positively, respectively.

Statistical analysis

Pearson χ^2 statistical analysis was performed to assess FGFR4 protein expression in the subgroups with differing clinicopathological characteristics. The probability of survival for different subgroups was calculated using the Kaplan-Meier method and the survival curves were plotted using the log rank test. All statistics were performed using 2-sided analysis, with a significance level of $P < 0.05$, using the "SPSS 19.0" statistical software package.

RESULTS

FGFR 4 protein expression

According to the criteria described previously, among 175 cases, 77 (44%) had high FGFR4 protein expression (Figure 1A) and 98 (56%) had low expression (Figure 1B).

Correlation of FGFR4 protein expression with clinicopathological characteristics

A significant correlation was observed between FGFR4 protein expression and Topo Ⅱα expression in gastric cancers (log rank χ^2 = 9.4760, P = 0.0236). No relationships were observed between FGFR4 expression and gender, age, tumor size, tumor location, tumor differentiation, macroscopic type, p53 status, p27 status or TNM GC classification ($P > 0.05$; Table 1). Furthermore, within the subgroups, no relationships were observed between FGFR4 protein expression and depth of invasion, lymph node metastasis, distant metastasis, neural invasion or vascular invasion (Table 1).

Survival analysis

The five-year survival rate for patients with tumors showing low expression of FGFR4 was 39.2%, and the median survival time was 39 mo. The five-year survival rate for patients with tumors showing high FGFR4 expression was 30.8%, and median survival time was 27 mo. However, analysis of the entire patient cohort using Kaplan-Meier survival analysis showed no difference in survival between patients with high and low FGFR4 expressing tumors (log rank χ^2 = 1.0477, P = 0.3060). When the patient population was stratified by clinicopathological parameters, such as age at diagnosis, gender, tumor size, differentiation, pathological stage, neural or vascular invasion, we found that the expression of FGFR4 protein was associated with a shorter survival time in GC patients if the tumor was small (less than or equal to 3 cm in size) (log rank χ^2 = 5.5033, P = 0.0190, Figure 2A), well differentiated (log rank χ^2 = 7.9757, P = 0.0047, Figure 2B), or of T1 or T2 stage (log rank χ^2= 4.8827, P = 0.0271, Figure 2C). No survival dif-

Table 2 Relationship between different clinicopathological characteristics and prognosis

Clinicopathological characteristics	FGFR4 positive		FGFR4 negative		χ^2	*P* value
	Median survival time (mo)	5-yr survival rate	Median survival time (mo)	5-yr survival rate		
Gender						
Male	27	27.4	47	36.7	1.1198	0.2900
Female	43	42.2	46	44.8	0.0000	0.9967
Age (yr)						
< 60	31	23.9	35	30.0	0.4080	0.5230
⩾ 60	44	36.1	52	46.0	0.6206	0.4308
Tumor size						
⩽ 3 cm	42	40.4	60	69.0	5.5033	0.0190
> 3 cm	22	24.8	24	26.2	0.0746	0.7848
Tumor location						
U	18	31.1	38	31.4	0.7164	0.3973
M	43	31.5	46	38.1	0.0225	0.8807
L	35	29.1	52	42.5	1.1274	0.2883
Tumor differentiation						
Poor	17	23.4	35	20.1	2.2622	0.1326
Well	31	47.0	56	62.1	7.9757	0.0047
TNM stages						
Ⅰ + Ⅱ	56	57.7	60	69.6	1.3128	0.2519
Ⅲ + Ⅳ	15	3.5	19	10.6	0.5328	0.4654
T						
T1 + T2	54	55.8	60	82.1	4.8827	0.0271
T3 + T4	22	19.4	18	21.3	0.0479	0.8268
N						
N0	58	67.4	60	75.0	0.4856	0.4859
N1 + N2 + N3	18	10.1	25	24.3	2.5385	0.1111
M						
0	43	35.3	52	45.4	1.2078	0.2718
1	9	0.0	12	0.0	0.4042	0.5249
Neural Ⅰ invasion						
Yes	19	38.9	56	18.8	2.1949	0.1385
No	36	30.2	49	43.3	3.1118	0.0777
Vascular Ⅰ invasion						
Yes	21	13.8	22	14.3	0.0507	0.8219
No	42	34.0	54	46.2	1.7283	0.1886

FGFR4: Fibroblast growth factor receptor 4; TNM: Tumor-node-metastasis.

ferences were observed in any of the other subgroups (Table 2).

DISCUSSION

In this single-center study, we investigated the FGFR4 protein expression status of 175 resectable GC specimens using IHC analysis. Herein, we focused on the role of FGFR4 as a prognostic marker for predicting cancer behavior and clinical outcome in GC patients undergoing curative surgery. To our knowledge, this is the largest study conducted to date.

Our data showed that 44% of cases (77) exhibited high FGFR4 protein expression, a result which is in keeping with a similarly high expression rate of around 38%, documented in a previous study which also reported that GC tissues have higher FGFR4 protein expression than normal tissues[13]. Overexpression of FGFR4 protein has been described in various malignancies and has been shown to play an important biological role. Roidl *et al*[14] in 2009 demonstrated that FGFR4 expression is up-regulated in response to doxorubicin treatment in apoptosis-resistant cancer cell clones. Turkington *et al*[15] in 2014 demonstrated that FGFR4 has an important role in resistance to oxaliplatin and 5-FU treatment in a range of colorectal cancer cell line models, whilst Zaid *et al*[9] in 2013 demonstrated that gene silencing of FGFR4 and inhibition of ligand-receptor binding both significantly decreased ovarian tumor growth both *in vitro* and *in vivo*. Recently, a study using a combination of the FGFR4 inhibitor, PD173074, and 5-fluorouracil showed an anti-proliferative and pro-apoptotic effect in GC cells *in vitro*[16]. Targeting gastric cancers with high levels of FGFR4 protein expression may represent a new therapeutic modality.

In our 175 patient cohort, no relationships were observed between FGFR4 protein expression and age, gender, tumor location, tumor differentiation, macroscopic type, TNM classification or other clinicopathological characteristics ($P > 0.05$). This is in keeping with similar data from several published studies on GC, hepatocellular carcinoma[17] and other tumor types. Results from earlier studies also showed that FGFR4 expression correlated significantly with the expression of human epidermal receptor 2 (HER-2), p21, and proliferating cell nuclear antigen (PCNA)[13]. In our study, we found that FGFR4 expression correlated positively with Topo Ⅱα expression, but not with p53 or p27.

Topo Ⅱα is a nuclear enzyme which modulates the topology of chromosomal DNA by causing transient double-stranded DNA breaks. This enzyme plays a key role in a number of DNA-related processes[18], is essential for cell growth and is typically expressed at high levels in rapidly growing cancer cells[19]. Notably, the fact that specific enzymatic inhibition of Topo Ⅱα results in significant antitumor activity confirms that Topo Ⅱα is an important target for anticancer agents[20]. Furthermore, reports have also shown that Topo Ⅱα is involved in multiple mechanisms of drug resistance in primary gastric cardiac adenocarcinoma[21]. Hence, we suggest that the correlation of high FGFR4 and Topo Ⅱα protein expression may, in part, explain the relatively poor prognosis for GC patients. Clearly, the underlying molecular mechanisms involved are complex and require further investigation.

In our study, the median survival time and 5-year survival rate for patients with high FGFR4 protein expression were both worse than those with low expression. However, no statistically significant differences were observed (log rank $\chi^2 = 1.0477$, $P = 0.3060$). These findings agree with the analysis of 94 GC patients performed by Ye *et al*[12] in 2012. We postulate that the up-regulation of FGFR4 may contribute to an antiapoptotic effect in GC cells[13], with

similar data reported in hepatocellular carcinoma[22] and colorectal cancer[23].

Notably, we observed significant statistical differences in FGFR protein expression following stratification of tumors by size, differentiation, and invasion depth ($P < 0.05$). The high expression of FGFR4 appears to play an important role in the prognosis of GC with fewer other risk factors including small tumor size, degree of differentiation and early stage invasive depth. Our results demonstrate that FGFR4 protein expression is a prognostic factor in relatively small (less than 3 cm), well-differentiated (grades Ⅰ and Ⅱ) and early stage invasive (stages Ⅰ and Ⅱ) GC tumors.

The role of FGFR4 as a cancer prognostic factor, however, still remains controversial. Li *et al*[24] in 2014 investigated 316 colorectal cancer cases and concluded that FGFR4 positivity was significantly correlated with shorter disease-free survival (DFS) and overall survival (OS). A further study by Brito *et al*[25] in 2012 demonstrated that FGFR4 protein overexpression and gene amplification were predictors of poor outcome in adult patients with adrenocortical tumors. In contrast, Dutra *et al*[26] in 2012 showed that low FGFR4 protein expression was related to lymph node positivity and premature relapse of disease, as well as disease-related death after analyzing 75 patients with squamous cell carcinoma of the mouth and oropharynx. Similar disagreement also occurs in GC. Ye *et al*[12] in 2012 analyzed 94 GC cases and subgroup analysis illustrated that in GC patients with Ⅲ/Ⅳ stage, the prognosis of patients with high expression of FGFR4 was much poorer. This is in contrast to the data presented here. We suggest that the smaller sample size in the study by Ye *et al*[12] may explain these conflicting results. Our study included a higher proportion of patients with large tumors and late-stage disease compared to the study by Ye *et al*[12]. A further possible explanation for this may be underestimation of the biomarker heterogeneity of GC. Clearly, further research with larger sample sizes is required to explain the full impact of FGFR4 on the development and prognosis of GC.

In conclusion, to date, this is the largest study focusing on the expression of FGFR4 protein in GC. Notably, our data show that high FGFR4 protein expression is related to the expression of Topo Ⅱα and poor overall survival in patients harboring relatively small (≤ 3 cm), well-differentiated tumors with early stage invasive depth. Overall, this study suggests that FGFR4 may represent an attractive therapeutic target in a subgroup of gastric cancers.

COMMENTS

Background

The fibroblast growth factor (FGF) pathway may represent an effective therapeutic option for cancer as FGF receptor 4 (FGFR4) protein expression is upregulated in several cancers. However, there are few studies on the role of FGFR4 in gastric cancer (GC).

Research frontiers

The authors investigated FGFR4 protein expression in Chinese patients with resectable GC and the association with clinicopathological characteristics and survival.

Innovations and breakthroughs

To date, this is the largest study focusing on the expression of FGFR4 protein in GC.

Applications

FGFR4 may be an effective therapeutic biomarker for GC. More studies should be performed to investigate the role of FGFR4 in GC.

Peer-review

This is an interesting article focusing on FGFR4 protein expression GC. They concluded that the FGFR4 high expression is not an independent risk factor for GC cancer initiation but that it is a useful prognostic marker for GC patients when the tumor is relatively small, well differentiated or in early depth invasion. More studies on the role of FGFR4 in GC should be performed.

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ORIGINAL ARTICLE

Retrospective Study

Retrospective analysis of extra-gastrointestinal stromal tumors

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Abstract

AIM: To investigate the clinicopathologic features of patients with extra-gastrointestinal stromal tumors (EGISTs) in South Korea.

METHODS: A total of 51 patients with an EGIST were identified. The clinicopathologic features, including sex, age, location, tumor size, histology, mitotic rate, immunohistochemical features, genetic status and survival data, were analyzed.

RESULTS: The median age was 55 years (range: 29-80 years), and male:female ratio was 1:1.04. The most common site was in the mesentery (n = 15) followed by the retroperitoneum (n = 13) and omentum (n = 8). The median tumor size was 9.0 cm (range: 2.6-30.0 cm) and the median mitotic rate was 5.0/50HPF. (1/50 - 185/50). *KIT* was analyzed in 16, which revealed 10 cases with wild-type *KIT* and 6 cases with an exon 11 mutation. Among 51 patients, 31 patients had undergone surgery, and 10 had unresectable disease and had taken palliative imatinib, which resulted in 22.7 mo of progression-free survival. Of the patients who had undergone surgery, 18 did not take adjuvant imatinib, and 8 of these were categorized as "high risk" according to the risk criteria. However, the relapse-free survival was not different (P = 0.157) between two groups.

CONCLUSION: Because the biologic behaviors of GISTs differ according to the location of the tumor, a more stratified strategy is required for managing EGISTs including incorporation of molecular features.

Key words: Gastrointestinal stromal tumor; Survival; Imatinib; Risk factor; Prognostic factor

Core tip: A gastrointestinal stromal tumor arising outside the gastrointestinal tract is called an extra-gastrointestinal stromal tumor (EGIST). In this study, we analyzed 51 patients with an EGIST and found that, patients with an EGIST have unique clinicopathologic features and distinct disease courses. Therefore, the risk stratification of this disease should be distinguished from that of GISTs.

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INTRODUCTION

A gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor of the gastrointestinal tract (GIT) with approximately 10 new cases diagnosed per 1 million each year[1-3]. Surgery is the only treatment leading to a potential cure, but more than 40% of cases recur and metastasize[4].

A GIST is thought to originate from the interstitial cells of Cajal (ICC), the pacemaker of the peristaltic movement of the GIT[5]. More than 95% of GISTs express the KIT protein, and recently DOG1 (discovered on GIST-1) has also been suggested as a useful diagnostic marker. These two immunohistochemical markers are considered to be the most specific and sensitive markers for GIST[1,6,7]. As for the genetic aberrations, approximately 80% of GISTs have a *KIT* mutation, and 8% to 10% have mutations in the gene encoding the platelet-derived growth factor receptor alpha polypeptide (*PDGFRa*). The gain-of-function mutations of those genes are critical in the carcinogenesis of GIST[8]. Thus, inhibitors of *KIT* and *PDGFRα*, such as imatinib[9], sunitinib[10] and regorafenib[11] are reasonable options for treatment.

The most common primary site of a GIST is the stomach (60% to 70%) followed by the ileum to jejunum (25% to 30%), the colorectum (5% to 15%), the duodenum (5%), and the esophagus (< 2%)[12,13]. The prognosis and genetic features are distinguishable according to the anatomical location; a gastric GIST has a better prognosis and a higher incidence of an exon 11 mutation of *KIT*, which is a favorable predictive marker for imatinib treatment, than that of a small intestinal GIST[13,14]. Some GIST develops outside the GIT, such as in the omentum, mesentery, and retroperitoneum, and this type of tumor is called an extra-gastrointestinal stromal tumor (EGIST)[12,15]. Although the incidence of EGISTs is reported to be approximately 10% of all GIST cases[16,17], the clinicopathologic parameters and clinical implications of an EGIST have yet to be defined because of the rarity of these tumors. Moreover, the role of imatinib, the drug of choice for this disease, is still unclear.

In the current study, we analyzed the clinicopathologic features of patients with an EGIST from multiple institutes in South Korea.

MATERIALS AND METHODS

Patients

Patients who were diagnosed with an EGIST from 2004 to 2012 were included in the analysis. The inclusion criteria were as follows; (1) a pathologically confirmed diagnosis of a GIST; (2) tumors that arose outside the GIT; and (3) a complete medical record, including demographics, site of primary tumor and pathologic reports. Patients with tumors that were attached to the serosa of the GIT, as determined by either radiologic or surgical field findings, were excluded.

Clinicopathologic parameters

We retrospectively collected clinicopathologic parameters from patients' medical records, including age, sex, primary tumor site, tumor size, histology, mitotic rate (per 50 high-power fields, HPF, 400 × magnification level), histologic grade, immunohistochemical findings (KIT, CD34, DOG1), genetic analysis (*KIT*, *PDGFRα*), use of imatinib and the date of surgery, recurrence, progression and death. Mutations analysis was done in exons 9, 11, 13, and 17 of the *KIT* gene and those of exons 12 and 18 of the *PDGFRα* gene. The analysis of mutations *via* the polymerase chain reaction amplification of genomic DNA for the *KIT* gene (exons 9, 11, 13 and 17) and the *PDGFRα* gene (exons 12 and 18) was performed as previously described[18,19].

Overall survival (OS) was measured from the date of diagnosis of the GIST to the date of death or last follow-up. Relapse-free survival (RFS) was measured from the date of curative surgery to the date of recurrence or last follow-up. Progression-free survival (PFS) was measured from the date of diagnosis of a metastatic or unresectable GIST to the date of progressive disease, death or last follow-up. All of the survival parameters were calculated using the Kaplan-Meier method and were compared using a log-rank test. *P* values > 0.05 were considered statistically significant, and all the *P*-values corresponded to two-sided significance tests.

Table 1 Baseline characteristics of the patients *n* (%)

Age, median (range)	51 (29-80)
Sex	
Male	25 (49.0)
Female	26 (51.0)
Primary site	
Mesentery	15 (29.4)
Retroperitoneum	13 (25.5)
Omentum	8 (15.7)
Vagina	3 (5.9)
Liver	3 (5.9)
Ovary	2 (3.9)
Pancreas	2 (3.9)
Perianal area	2 (3.9)
Chest wall	1 (2.0)
Pleura	1 (2.0)
Prostate	1 (2.0)
Tumor size, median (range)	9.0 cm (2.6-30.0)
Mitotic rate[1], median (range)	5.0 (1-185)
Histologic morphology	
Epithelioid	15 (29.4)
Spindle	27 (52.9)
Pleomorphic	9 (17.7)
KIT IHC	
Positive	47 (92.2)
Negative	4 (7.8)
CD34 IHC (n = 31)	
Positive	25 (80.6)
Negative	6 (19.4)
DOG1 IHC (n = 13)	
Positive	13 (100.0)
Negative	0 (0.0)
KIT gene analysis (n = 16)	
Exon 11 mutation	6 (37.5)
Wild-type	10 (62.5)
PDGFRα gene analysis (n = 6)	
Exon 18 mutation	4 (66.7)
Wild type	2 (33.3)

[1]Per 50 high power fields. IHC: Immunohistochemistry.

RESULTS

Patient characteristics

A total of 51 patients from 7 institutes were found to be eligible for the analysis. The median age of patients was 51 years (range: 29-80 years) and male:female ratio was 1:1.04. The most common primary site was in the mesentery (n = 15) followed by the retroperitoneum (n = 13) and omentum (n = 8). Other primary sites were the vagina (n = 3), liver (n = 3), ovary (n = 2), pancreas (n = 2), perianal area (n = 2), chest wall (n = 1), pleura (n = 1) and prostate (n = 1). The median size of the tumor was 9.0 cm (range 2.6-30.0 cm), and the median mitotic rate per 50 HPF was 5.0 (range: 1-185). Regarding the morphology, 15 (29.4%) were epithelioid, 27 (52.9%) were spindle cell; and 9 (17.7%) were a pleomorphic type. On immunohistochemical analysis, KIT was positive in 47 (92.2%) cases; CD34 was positive in 25 cases (80.6%, 31 cases examined), and DOG1 was positive in 13 cases (100.0%, 13 cases examined). The 4 cases not expressing KIT were with *KIT* gene analysis. Regarding the genetic status, *KIT* analysis was performed in 16 cases with 10 (62.5%) cases of wild-type and 6 (37.5%) cases of exon 11 mutation identified. *PDGFRα* analysis was carried out in 6 cases among cases with wild-type *KIT*, and 4 (66.7%) of those examined had an exon 18 mutation. These are summarized in Table 1.

Hospital courses and survival analysis

Among 51 patients, 10 patients did not receive any type of treatment, and they were lost to follow up after the initial diagnosis. Out of the 41 remaining patients, 31 patients underwent a curative resection, 13 of whom received imatinib as an adjuvant treatment and 18 of whom did not receive adjuvant treatment. Ten of the 41 were diagnosed with metastatic or unresectable disease at the time of diagnosis and received imatinib as palliative treatment.

Regarding the histologic features of the patients who had undergone curative resection, the physicians used somewhat different criteria for imatinib treatment compared to the NIH criteria[20]. The histologic features for the 13 patients who were treated with adjuvant imatinib were as follows: median tumor size was 11.0 cm (range: 3.0-25.0 cm); the median mitotic rate was 7.0 per 50 HPF (range: 2-185 per 50 HPF); and the median RFS was 60.1 mo. The histologic features for the 18 patients who did not receive adjuvant imatinib after surgery were as follows; the median tumor size was 6.5 cm (range: 2.6-18.0 cm); the median mitotic rate was 3.0 per 50 HPF (range: 0-45 per 50 HPF); and the median RFS could not be calculated because of the small number of event (n = 2). When we categorized these patients according to the NIH criteria for risk of recurrence, 10 patients were categorized as "low-intermediate risk", whereas 8 patients fell into the "high risk" group. However, there was no difference in the RFS between two groups (P = 0.157). When separating the two prognostic factors, a mitotic rate higher than 5/50 HPF showed a trend to predict more recurrence (P = 0.061), but this result did not reach statistical significance, and a tumor size > 5.0 cm was not associated with the risk of recurrence (P = 0.866).

Regarding the 10 patients who were treated with imatinib as a palliative measure, the median tumor size was 16.0 cm (range: 4.0-30.0 cm), the median mitotic rate was 10.0 per 50 HPF (range: 3-60), and the median PFS was 22.7 mo (95%CI: 7.9-37.5). Median OS of these patients was 37.6 mo (95%CI: 0.3-94.5).

The hospital courses of the patients and their clinicopathologic features are described in Figure 1.

DISCUSSION

In the current study, we analyzed the clinicopathologic features of 51 patients with EGISTs across 7 institutes. The demographics such as age and the sex ratio were similar to those in patients with stromal tumors that had arisen inside the GIT. And with a GIST, the KIT-

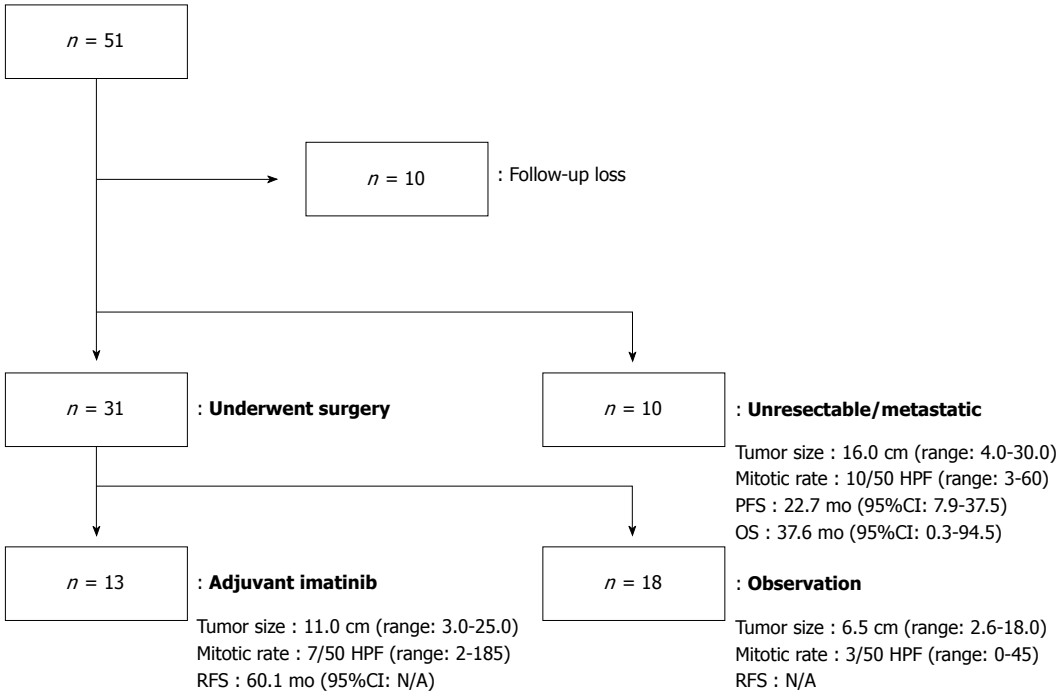

Figure 1 Hospital courses and clinicopathologic features of the patients. The tumor size, mitotic rate and survival data are presented as median values. HPF: High-power field; PFS: Progression free survival; OS: Overall survival; RFS: Relapse free survival; CI: Confidence interval; N/A: Not available.

positive rate was over 90% (47/51), and DOG1-positive rate was 100% (13/13). With regard to the genetic status, however, rate of KIT wild-type was higher than expected (67.5%). Imatinib achieved median PFS of 22.7 mo which is comparable to that of advanced GIST patients who were treated with the drug[21,22].

Compared to a GIST, the prognosis of EGIST is known to be less favorable[15,17]. This is assumed because an EGIST harbors poor prognostic factors, including high proliferative indices, a large tumor size, lymph node involvement, and distant metastasis. Because development outside GIT may result in a delay of the presentation of clinical symptoms, a considerable portion of EGIST cases are diagnosed at a late stage, which can make it difficult to manage the case surgically and thereby results in worse clinical outcomes. In contrast, there are several reports that tumor size does not impact the prognosis of EGIST patients. Reith *et al*[15] found that a tumor size larger than 10.0 cm dose not influence the clinical outcomes of 48 patients with EGIST. Furthermore, in Yamamoto's report, tumor size did not correlate with patient survival[23]. However, in these two studies, proliferation indices, such as the mitotic rate, cellularity and Ki-67 expression, were shown to be prognostic factors for survival.

In the current study, we observed similar results. Although tumor size was not associated with survival, the mitotic rate showed a tendency to be associated with survival. Because a substantial portion of EGISTs are diagnosed with a large tumor size, it is possible that tumor size itself may not reflect the biology of the EGIST. Because the tumor size has different clinical implications to the anatomical sites[13], the prognostic role of tumor size in EGISTs requires further analysis.

Approximately two-thirds of patients with a conventional GIST have a *KIT* mutation at exon 11[3]. Regarding an EGIST, the incidence of this type of mutation is reported to be approximately 40%-50%, which is somewhat lower[16,23]. In the present study, we found 6 cases with an exon 11 mutation out of 16 patients (37.5%). According to these results, it appears that EGIST patients less frequently harbor an exon 11 mutation. As this mutation is indicative of a good response to imatinib, further analysis with a greater number of cases is required.

Surgery has been the frontline treatment of an EGIST[24-30]. After surgery, the administration of imatinib usually follows according to the NIH criteria, which are determined by the tumor size, mitotic rate and anatomic location. In the current study, physicians did not strictly apply these criteria. Although the median tumor size (11.0 cm *vs* 6.5 cm) and the median mitotic rate (7.0/50 *vs* 3.0/50) were higher in patients who were administered imatinib (n = 13) than those of the patients who were only observed after surgery (n = 18), 8 out of 18 patients should have nevertheless been treated with imatinib according to the NIH criteria. However, as previously mentioned, RFS was not different (P = 0.157).

Several hypotheses have been suggested for the carcinogenesis of an EGIST. The tumor is identical to a GIST regarding the histologic, immunohistochemical

and genetic features[12,15,23]. Because the presence of interstitial Cajal-like cells has been reported in many organs outside the GIT, it is rational to suppose that an EGIST originates from common precursor cells that differentiate into the ICC-derived neoplasm during their development outside of the GIT. Another hypothesis is that this tumor might come from pluri-potential stem cells located outside the GIT. The extramural extension of a stromal tumor within the GIT is another hypothesis.

We reported an analysis of the clinicopathologic features and hospital courses of 51 patients with an EGIST. Considering the distinct features of EGISTs, a more precise strategy is required for managing this tumor.

COMMENTS

Background

Gastrointestinal stromal tumors (GISTs) constitute approximately 1% of tumors of the gastrointestinal tract. A curative surgical resection and the use of *KIT* inhibitors are the most important treatment modalities. This type of tumor sometimes develops outside the alimentary canal and is called an extra-gastrointestinal stromal tumor (EGIST).

Research frontiers

There are several studies suggesting that the clinicopathologic and molecular features differ between a GIST and an EGIST. Because the anatomic location, histologic features and genetic status are well-known risk factors for a GIST, understanding these features of an EGIST may help us to treat this disease.

Innovations and breakthroughs

The current study is one of the largest analyses dealing with patients with EGIST, and as with other studies, the authors have found that the tumor size itself was not associated with survival. The authors are one of the first to show that the clinical outcomes of imatinib treatment in patients with an EGIST are comparable to those of patients with a GIST.

Applications

This study suggests that different risk criteria may be applied when making clinical decisions for patients with an EGIST.

Peer-review

Authors evaluate the efficacy of strategies including surgery and treatment with imatinib in patients suffering from extra-gastrointestinal stromal tumors. This work adds new data of interest to establish the more appropriate treatment of these tumors. This paper can be accepted after minor revision.

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ORIGINAL ARTICLE

Retrospective Study

Tumor regression grades: Potential outcome predictor of locally advanced rectal adenocarcinoma after preoperative radiotherapy

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Abstract

AIM: To analyze tumor regression grade (TRG) for prognosis of locally advanced rectal adenocarcinoma (LARA) treated with preoperative radiotherapy.

METHODS: One hundred and ninety patients with clinical stage Ⅱ/Ⅲ LARA were studied. All patients underwent radical surgery (between 2004 and 2010) after 30-Gy/10-fraction preoperative radiotherapy (pre-RT). All 190 patients received a short course of pre-RT and were reassessed for disease recurrence and survival; the slides of surgical specimens were reviewed and classified according to Mandard TRG. We compared patients with good response (Mandard TRG1 or TRG2) *vs* patients with bad/poor response (Mandard TRG3-5). Outcomes evaluated were 5-year overall survival (OS), 5-year disease-free survival (DFS), and local, distant and mixed recurrence. Fisher's exact test or χ^2 test, log-rank test and proportional hazards regression analysis were used to calculate the probability that Mandard TRG was associated with patient outcomes.

RESULTS: One hundred and sixty-six of 190 patients (87.4%) were identified as Mandard bad responders (TRG3-5). High Mandard grade was correlated with tumor height (41.7% < 6 cm *vs* 58.3% ⩾ 6 cm, P = 0.050), ypT stage (75% ypT0-2 *vs* 25% ypT3-4, P = 0.000), and ypN stage (75% ypN0 *vs* 25% ypN1, P = 0.031). In univariate survival analysis, Mandard grade bad responders had significantly worse OS and DFS

than good responders (TRG1/2) (OS, 83.1% *vs* 96.4%, *P* = 0.000; DFS, 72.3% *vs* 92.0%, *P* = 0.002). In multivariate survival analysis, Mandard bad responders had significantly worse DFS than Mandard good responders (DFS 3.8 years (95%CI: 1.2-12.2 years, *P* = 0.026).

CONCLUSION: Mandard grade good responders had a favorable prognosis. TRG may be a potential predictor for DFS in LARA after pre-RT.

Key words: Tumor regression grade; Preoperative radiotherapy; Rectal adenocarcinoma; Disease-free survival

Core tip: We report that Mandard tumor regression grade (TRG) predicted the outcome of locally advanced rectal adenocarcinoma after preoperative radiotherapy. We examined the correlation of TRG in the prognosis of rectal adenocarcinoma. We found that high Mandard grade was correlated with tumor height, ypT stage, and ypN stage in Mandard poor responders. In univariate survival analysis, Mandard bad responders had significantly worse overall survival and disease-free survival (DFS) compared with Mandard good responders. In multivariate survival analysis, Mandard bad responders had significantly worse DFS than Mandard good responders. Mandard good responders had a favorable prognosis.

Peng YF, Yu WD, Pan HD, Wang L, Li M, Yao YF, Zhao J, Gu J. Tumor regression grades: Potential outcome predictor of locally advanced rectal adenocarcinoma after preoperative radiotherapy. *World J Gastroenterol* 2015; 21(6): 1851-1856 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i6/1851.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i6.1851

INTRODUCTION

Rectal cancer is a worldwide health concern[1]. In China, the incidence of rectal cancer is increasing at a rate of 4.2% annually, and is the fifth leading cause of cancer mortality[2]. Surgery remains the primary therapeutic tool for rectal cancer, and locoregional recurrence has been reduced by total mesorectal excision (TME) of cancers of the middle and lower rectum[3]. Preoperative radiotherapy (pre-RT), including short- or long-term courses, followed by TME can induce tumor regression and facilitate subsequent resection, resulting in improved local control and survival[4].

Although short- and long-term pre-RT regimens are considered standard for rectal cancer in western countries, they are not widely recommended in Asian countries such as Japan and China, where surgeons are more likely to perform extended operations to minimize local recurrence[5]. In China, a modified 30-Gy protocol for pre-RT was recommended by the Chinese Anti-Cancer Association in 2001 to minimize side effects and to increase flexibility without compromising therapeutic efficacy[6]. Our previous study[6] showed that, compared with surgery alone, the modified 30-Gy protocol was associated with significantly reduced local recurrence and complication rates. Patients had improved survival and downstaging, and clinical outcome was equivalent to standard pre-RT regimens. However, pre-RT (including the modified 30-Gy protocol) does not achieve benefit in all patients[4]. To quantify the response to pre-RT, different systems can be used that are particularly important in situations where the pathological response is not complete. Most of them have a 5-grade system, allowing the creation of groups according to the response[7,8]. In the present study, we aimed to assess the prognostic value of Mandard tumor regression grade (TRG)[9] in patients with locally advanced rectal adenocarcinoma (LARA) treated with 30-Gy/10-fraction pre-RT.

MATERIALS AND METHODS

Ethics

This study was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. This study was approved ethically by Peking University Cancer Hospital Institutional Review Board. All patients provided written informed consent.

Clinical data

Data from 190 patients with resectable rectal adenocarcinoma treated in our hospital from June 2004 to August 2010 were collected. Eligible patients were selected according to the following criteria[10,11]: (1) resectable rectal cancer ⩽ 10 cm from the anal verge; (2) evaluated by endorectal ultrasound (ERUS) or magnetic resonance imaging (MRI) before treatment; (3) primary carcinoma of the rectum identified histologically; (4) no clinical evidence of distant metastases; (5) having undergone transabdominal radical resection based on the principle of TME; and (6) having undergone R0 resection. Exclusion criteria were as follows: (1) patients who underwent concurrent neoadjuvant radiochemotherapy; (2) synchronous tumors or history of other malignant tumors within 5 years; (3) familial adenomatous polyposis and/or hereditary non-polyposis colorectal carcinoma; and (4) patients who died of complications or other non-cancer-related reasons.

Pretreatment evaluation, neoadjuvant therapy and surgery

All included patients underwent ERUS or MRI to evaluate tumor size, invasion depth and extent (T stage). All patients were identified as having involvement of the

Table 1 Clinical parameters

Variables	*n* (%)
Sex	
Male	118 (62.1)
Female	72 (37.9)
Age (yr)	
< 65	129 (67.9)
⩾ 65	61 (32.1)
Tumor height (cm)	
< 6	113 (59.5)
⩾ 6	77 (40.5)
Pre-TNM	
Ⅱ	30 (15.8)
Ⅲ	160 (84.2)
Pre-RT	190
Surgical procedure	
LAR	127 (66.8)
APR	58 (30.6)
Other	5 (2.6)
Perioperative complications	
Morbidity	43 (22.6)
Abdominal or pelvic abscess	8
Anastomosis leak	11
Reoperation	11

TNM: Tumor node metastasis; Pre-RT: Preoperative radiotherapy.

pararectal lymph nodes and were diagnosed as clinical stage Ⅱ/Ⅲ rectal cancer. Serum carcinoembryonic antigen was measured, and abdominal computed tomography (CT) and chest radiography were also routinely performed before treatment. Pre-RT with a total dose of 30 Gy/10 fractions was adopted, as recommended by the Chinese Anti-Cancer Association, based on high-level clinical evidence[12-14]. Surgical resection was performed 2-4 wk after full-dose RT.

TRG grade

Standard pathological tumor staging of the resected specimen was performed in accordance with the guidelines of the American Joint Committee on Cancer. Evidence of pathological complete response (ypCR) was defined as absence of viable adenocarcinoma in the surgical specimen or the presence of lakes of mucus without tumor cells. The histology of all surgical specimens was reviewed and confirmed independently and was classified based on the Mandard TRG system[9]. Grade 1: complete regression (fibrosis without detectable tumor tissue); Grade 2: fibrosis with scattered tumor cells; Grade 3: fibrosis and tumor cells with preponderance of fibrosis; Grade 4: fibrosis and tumor cells with preponderance of tumor cells; and Grade 5: tumor tissue without changes of regression.

Postoperative therapy, follow-up and endpoint

All patients in the pre-RT group were given adjuvant chemotherapy for six to eight cycles, using the standard regimens based on 5-fluorouracil or capecitabine, such as FOLFOX, CapeOX, or capecitabine alone. Patients were followed at 3-mo intervals for the first 2 years and then at 6-mo intervals for the next 3 years. Evaluations consisted of physical examination, serum carcinoembryonic antigen, complete blood count, and blood chemical analysis. Proctoscopy, abdominal ultrasonography, CT of the abdomen and pelvis, and chest radiography were also routinely performed every 6-12 mo. Endpoints of this study were 5-year overall survival (OS) and 5-year disease-free survival (DFS).

Statistical analysis

Statistical analyses were performed using SPSS version 16.0 software. The categorical variables were analyzed with Pearson χ^2 or Fisher's exact test. Survival curves were plotted using the Kaplan-Meier method, and log-rank tests were performed to evaluate prognostic differences between groups. The Cox proportional hazards model was used for multivariate analysis. For all analyses, two-sided tests of significance were used, and $P < 0.05$ was considered significant.

RESULTS

Patient characteristics

We studied 190 patients (118 male, 62 female) with mid-low rectal adenocarcinoma treated with pre-RT. The median patient age was 58 years (range: 28-85 years). The median distance of the tumor from the anal verge was 5 cm (range: 1-10 cm). One hundred and twenty-nine patients had sphincter preservation, while the other 58 received abdominoperineal resection, and three underwent the Hartmann procedure. The morbidity of the series was 22.6% (Table 1).

Response to pre-RT (30 Gy/10 fractions)

The distribution of the proportions of ypTNM stages (Table 2) were as follows: complete response (no microscopic residual tumor cell), 2.6% (n = 5); Stage Ⅰ, 25.8% (n = 49); Stage Ⅱ, 27.4% (n = 52); and Stage Ⅲ, 44.2% (n = 84). The median follow-up duration was 56 mo (range 3-125 mo), and the follow-up rate was 100%. Response to neoadjuvant therapy is outlined in Table 2. Classification of TRG according to the Mandard system allowed us to define two groups as previously described[3]: TRG1/2 and TRG3-5. We verified a good response to 30 Gy/10 fractions pre-RT in 24 patients (ypCR in 5%-12.6%) and a bad response in 166 patients (87.4%). The two groups of patients (good *vs* bad Mandard response) were comparable with respect to age (P = 0.284), sex (P = 0.379), clinical stage (P = 0.547), and surgical procedures performed (P = 0.173), with the exception of tumor height (P = 0.050), ypN-stage (ypN0/ypN+) (P = 0.031), and ypT-stage (ypT0-2/ypT3-4) ($P < 0.001$) (Table 3).

Disease recurrence

Seventeen patients (8.9%) had local recurrence, 66 (34.7%) had distant recurrence, and seven (3.7%)

Table 2 Pathological parameters and clinical long-term outcome *n* (%)

Variables	
Postoperative stage	
0	5 (2.6)
I	49 (25.8)
II	52 (27.4)
III	84 (44.2)
Mandard TRG	
Good response (1 or 2)	24 (12.6)
Bad response (3-5)	166 (87.4)
Overall recurrence of disease	
Local	17 (8.9)
Distant	66 (39.8)
Local and distant	7 (3.7)
5-yr OS	65.3% ± 2.2%
5-yr DFS	61.4% ± 4.4%

OS: Overall survival; DFS: Disease-free survival; TRG: Tumor regression grades.

Table 3 Comparison between tumor regression grades and demographic and clinic variables *n* (%)

Parameter	TRG1 + 2	TRG3 + 4 + 5	*P* value
Sex			
Male	17 (70.8)	101 (60.8)	0.379
Female	7 (29.2)	65 (39.2)	Fisher's Test
Age (yr)			
< 65	15 (62.5)	114 (68.7)	0.284
⩾ 65	9 (37.5)	52 (32.3)	Fisher's Test
Tumor height (cm)			
< 6	10 (41.7)	104 (62.7)	0.050[1]
⩾ 6	14 (58.3)	62 (27.3)	χ^2 =3.847
Clinical stage			
II	5 (20.8)	25 (15.1)	0.547
III	19 (79.2)	141 (84.9)	Fisher's Test
Surgical procedure			
LAR	20 (83.3)	107 (64.5)	0.173
APR + other	4 (16.7)	59 (35.5)	Fisher's Test
Pathological N-stage			
ypN0	18 (75.0)	85 (51.2)	0.031[1]
ypN1	6 (25.0)	81 (48.8)	Fisher's Test
Pathological T-stage			
ypT0-2	18 (75.0)	47 (28.3)	0.000[1]
ypT3-4	6 (25.0)	119 (71.7)	Fisher's Test

[1]Fisher's exact test 2-sided. TRG: Tumor regression grades.

had mixed recurrence.

Survival analysis

In univariate analysis, the mean follow-up was 56 mo (range: 3-125 mo). The 5-year OS and DFS was 65.3% and 61.4%, respectively (Table 2). In the different subsets, survival at 5 years was matched (Table 4). The 5-year OS and DFS in the patients who showed a bad and good response on Mandard TGR were 96.4% *vs* 83.1% (P = 0.002) and 92.0% *vs* 72.3% (P < 0.000), respectively (Table 4 and Figure 1). In multivariate Cox regression, DFS (3.8 years, 95%CI: 1.2-12.2 years, P = 0.026) in patients with bad Mandard response was significantly worse than

Table 4 Tumor regression grade and clinical long-term outcome

Variables		*P* value
5-yr OS		
Mandard good response (TRG1/2)	96.4% ± 2.0%	0.002[1]
Mandard good response (TRG3-5)	83.1% ± 4.2%	
ypCR (Mandard TRG1)	100.00%[2]	0.691[1]
Mandard partial response (TRG2)	86.68%[2]	
5-yr DFS		
Mandard good response (TRG1/2)	92.0% ± 3.5%	0.000[1]
Mandard good response (TRG3-5)	72.3% ± 3.8%	
ypCR (Mandard TRG1)	100.00%[2]	0.502[1]
Mandard partial response (TRG2)	86.68%[2]	

[1]Log rank test; [2]No statistics were computed because all cases were censored. Univariate analysis follow-up: mean 56 mo (range: 3-125 mo). OS: Overall survival; DFS: Disease-free survival; TRG: Tumor regression grades.

in those with a good response after the following variables were entered: ypN stage (ypN0/ypN$^+$), ypT stage, and tumor height. There was no significant survival difference in OS (6.5 years, 95%CI: 0.9-48.2 years, P = 0.066) between patients with bad and good Mandard response when comparing patients with complete (ypCR or Mandard TRG1) and partial (Mandard TRG2) pathological response (OS, P = 0.691; DFS, P = 0.502) (Table 4).

DISCUSSION

Neoadjuvant therapy, whether chemo-RT or RT alone, shows a significant improvement in local control and sphincter preservation[11,14-16]. Histological changes after pre-RT for rectal carcinoma vary considerably, with some entities showing complete absence of tumor cells, whereas others exhibit a mass of tumor cells with little or no regressive changes[17,18]. Compared with tumor downstaging or volume shrinkage, TRG can more accurately reflect tumor response at a cellular level[8]. Unlike the United States, in China, modified pre-RT (30-Gy/10 fractions) is widely adopted[19], thus our study addressed the clinical value of TRG following this particular RT regimen.

Our data confirmed that histological regression is closely coordinated with pathological T and N stage. As stated above, a significant proportion of cases with poor histological response contributed to the nodal-positive group, which indicated that TRG is an effective supplement to the TNM classification. A majority of studies support the view that patients with complete or partial response to preoperative treatment had better DFS than those with poor response[17,20,21]. Our data further demonstrated this conclusion by dividing TRG into poor response and TRG into good response in the Mandard system. This could guide the clinical decision making for postoperative adjuvant chemotherapy. For example, among patients with stage II rectal cancer who had no response, intensive chemotherapy might be considered in the adjuvant

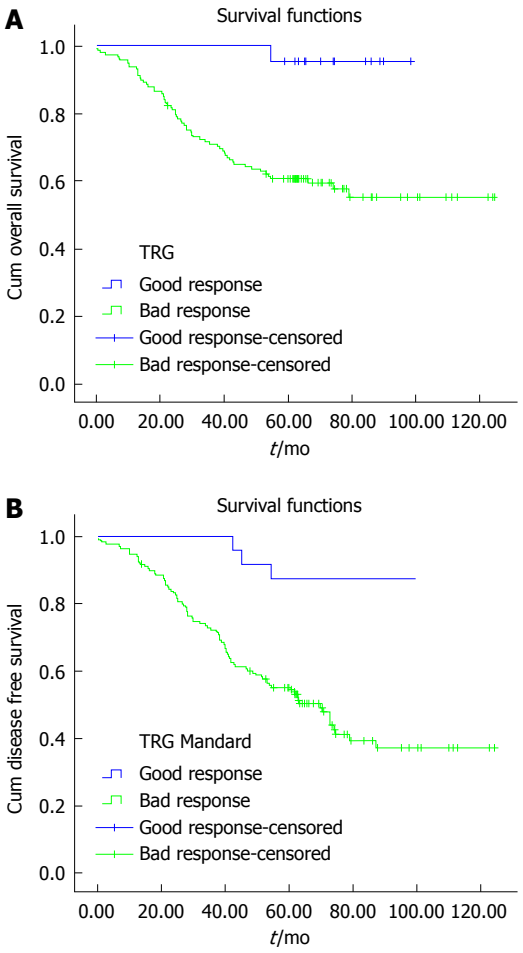

Figure 1 Kaplan-Meier survival curves were plotted to predict survival. Five-year OS (A) and 5-year DFS (B) in the two Mandard groups. OS: Overall survival; DFS: Disease-free survival; TRG: Tumor regression grades.

setting. In contrast, patients with a partial, but not complete response might not need further adjuvant chemotherapy and may benefit from fewer adverse drug effects.

Although the prognostic value of TRG has been well demonstrated, the clinical application of TRG still faces many problems. First, there are several TRG systems besides Mandard, and each has its own characteristics and indications[22,23]; however, which TRG system is more suitable for China is still in question. Our study suggests that the Mandard system is a potential candidate for tumor regression evaluation. Second, some studies have reported that the prognostic significance of TRG is not as crucial as ypTNM stage[24]. Thus, the association and role of TRG and TNM classification in prognostic evaluation needs to be addressed further.

Our study had some limitations. First, this study was retrospective, thus it might have had selection bias. Second, we did not compare the efficiency of the Mandard and other TRG systems in predicting tumor progression, thus, we could not conclude whether other TRG systems were better than Mandard. Third, the case number in some TRG subgroups was small, which may have influenced the reliability of the statistics.

In summary, our study demonstrates that TRG is a significant prognostic system for tumor progression and survival. It is a promising criterion for clinical decision making in adjuvant therapy.

COMMENTS

Background

This study showed that Mandard tumor regression grade (TRG) predicted the outcome of locally advanced rectal adenocarcinoma (LARA) after 30-Gy/10-fraction preoperative radiotherapy (pre-RT).

Research frontiers

Neoadjuvant therapy, whether chemo-RT or RT alone, results in a significant improvement in local control and sphincter preservation. Histological changes after pre-RT for rectal carcinoma vary considerably, with some entities showing complete absence of tumor cells, whereas others exhibit a mass of tumor cells with little or no regressive changes. Compared with tumor downstaging or volume shrinkage, TRG could more accurately reflect tumor response at a cellular level.

Innovations and breakthroughs

The authors examined the correlation of TRG in the prognosis of rectal adenocarcinoma. They found that high Mandard grade was correlated with tumor height, ypT stage, and ypN stage in Mandard poor responders. In univariate survival analysis, Mandard bad responders had significantly worse overall survival and disease-free survival (DFS) than Mandard good responders (TRG1/2). In multivariate survival analysis, Mandard bad responders had significantly worse DFS than Mandard good responders. Mandard good responders had a favorable prognosis. Together, these studies suggest that TRG may be used as a potential predictor for DFS in Stage Ⅱ/Ⅲ LARA after short-course RT.

Applications

Mandard good responders had a favorable prognosis. TRG may be used as a potential predictor for DFS in Stage Ⅱ/Ⅲ LARA after 30-Gy/10-fraction pre-RT.

Terminology

The TRG was first introduced by Mandard *et al* following the management of a patient with esophageal cancer. The TRG can categorize the cancer cell ratio from tissue with fibrosis or inflammation in numerous stages throughout chemoradiotherapy. TRG was quantitated in five grades: TRG 1 (complete regression) showed absence of residual cancer and fibrosis extending through the different layers of the esophageal wall; TRG 2 was characterized by the presence of rare residual cancer cells scattered through the fibrosis; TRG 3 was characterized by an increase in the number of residual cancer cells, but fibrosis still predominated; TRG 4 showed residual cancer outgrowing fibrosis; and TRG 5 was characterized by the absence of regressive changes.

Peer-review

This is a very interesting study about Mandard TRG in patients with stage Ⅱ-Ⅲ LARA. In this manuscript, the authors analyzed the prognostic value of Mandard TRG in patients with stage Ⅱ-Ⅲ locally advanced rectal adenocarcinoma treated with 30-Gy/10-fraction preoperative radiotherapy followed by radical surgery.

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ORIGINAL ARTICLE

Retrospective Study

Prediction of synchronous colorectal cancers by computed tomography in subjects receiving an incomplete colonoscopy: A single-center study

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Abstract

AIM: To assess the value of computed tomography (CT) for diagnosis of synchronous colorectal cancers (SCRCs) involving incomplete colonoscopy.

METHODS: A total of 2123 cases of colorectal cancer (CRC) were reviewed and divided into two groups according to whether a complete or incomplete colonoscopy was performed. CT results and final histological findings were compared to calculate the sensitivity and specificity associated with CT for detection of SCRCs following complete *vs* incomplete colonoscopy. Factors affecting the CT detection were also analyzed.

RESULTS: Three hundred and seventy-four CRC patients underwent incomplete colonoscopy and 1749 received complete colonoscopy. Fifty-six cases of SCRCs were identified by CT, and 36 were missed. In the incomplete colonoscopy group, the sensitivity and specificity of CT were 44.8% and 93.6%, respectively. The positive and negative predictive values were 23.6% and 95.0%, respectively. In contrast, the sensitivity and specificity of CT for the complete colonoscopy group were 68.3% and 97.0%, while the positive and negative predictive values were 22.2% and 98.7%, respectively. In both groups, the mean maximum dimension of the concurrent cancers identified in the CT-negative cases was shorter than in the CT-positive cases (incomplete group: $P = 0.02$; complete group: $P < 0.01$) Topographical proximity to synchronous cancers was identified as a risk factor for missed diagnosis ($P = 0.03$).

CONCLUSION: CT has limited sensitivity in detecting SCRCs in patients receiving incomplete colonoscopy.

Patients with risk factors and negative CT results should be closely examined and monitored.

Key words: Synchronous colorectal cancer; Computed tomography; Colonoscopy

Core tip: This retrospective study aimed to evaluate the diagnostic accuracy of computed tomography (CT) in predicting synchronous colorectal cancers, especially in patients with incomplete colonoscopy. The study suggested that CT remains a feasible option when complete colonoscopy cannot be performed. However, the sensitivity of CT is limited due to several factors such as small tumor size, lumen conditions, and tumor location.

Pang EJ, Liu WJ, Peng JY, Chen NW, Deng JH. Prediction of synchronous colorectal cancers by computed tomography in subjects receiving an incomplete colonoscopy: A single-center study. *World J Gastroenterol* 2015; 21(6): 1857-1864 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i6/1857.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i6.1857

INTRODUCTION

Cases of synchronous colorectal cancers (SCRCs) involve the detection of two or more primary colorectal carcinomas in a single individual upon an initial diagnosis of colorectal cancers (CRC). Currently, the Warren and Gates criteria[1] are used to diagnose SCRCs clinically. These criteria include the following key elements: (1) each tumor must be proved to be malignant; (2) each tumor must be distinct; (3) the probability of one tumor being a metastasis of another must be excluded; and (4) the synchronous lesions must be diagnosed simultaneously or within 6 mo of initial diagnosis[2]. In cases of SCRCs, the most advanced cancer (as determined by TNM staging) is usually defined as the index cancer, while the less-advanced tumors are considered concurrent lesions to the index cancer (Figure 1).

SCRCs are of major clinical importance because a missed diagnosis of concurrent lesions can lead to remnant cancer. The prognosis of SCRCs is typically identical to that of solitary cancer[3,4] as long as any synchronous lesions present are correctly diagnosed prior to surgery. Colonoscopy is ideal for detecting synchronous lesions, except when bowel preparation is inadequate. Moreover, the presence of a scirrhous carcinoma may hinder the passage of an endoscope through the lumen[5]. As a result, incomplete colonoscopy due to these or other factors can result in failure to detect additional lesions. In developing countries, such as China, conventional computed tomography (CT) is typically used to examine the remnant bowel not examined by colonoscopy because CT colonography (CTC) is generally not available. Therefore, the goal of this study was to evaluate the diagnostic accuracy of CT in predicting cases of SCRCs, especially in patients that have undergone incomplete colonoscopy.

MATERIALS AND METHODS

Subjects

Between January 2002 and August 2011, 2186 patients with CRC were treated at the Shanghai Sixth People's Hospital. Sixty-three patients underwent colonoscopy or CT alone, and these cases were excluded. Cases of SCRCs were defined as patients with more than one primary colorectal adenocarcinoma present at the time of resection, or within 6 mo following resection. For each patient, the most advanced carcinoma according to T staging was identified as the index carcinoma, while the remaining less-advanced cancers were identified as concurrent carcinomas. If carcinomas with the same T stage were present, the lesion with the largest volume was identified as the index carcinoma. The remaining 2123 patients were divided into two groups according to those that underwent incomplete *vs* complete colonoscopy (Figure 2). The main reasons for incomplete colonoscopy included: colorectal obstruction, elongation of the colon, inadequate bowel preparation, intolerance to the procedure, and diverticular disease.

Imaging techniques

All patients included underwent preoperative CT using a Lightspeed VCT (GE Medical Systems, United States). Patients were examined in the supine position and received an intravenous injection of contrast medium. Images were obtained using a conventional method with 5-mm thick sections (120 KV, 350 mA). All of the images were retrospectively and independently reviewed by at least two radiologists with knowledge of each patient's history, clinical presentation, and colonoscopy outcome. However, the radiologists were blinded to any information regarding operative findings or final diagnosis. Final CT interpretations had to be approved by both radiologists. If there were significant discrepancies, the images in question would be submitted to two additional senior radiologists for evaluation in order to obtain a final conclusion.

Analysis and statistics

Detailed pathological characteristics of the concurrent lesions for this cohort were collected by two surgeons (RRS and QCZ), and these characteristics included: maximum lesion dimension, gross appearance, differentiation, and location. The pathological results were taken as the reference standard against which the CT findings were compared. Furthermore, the

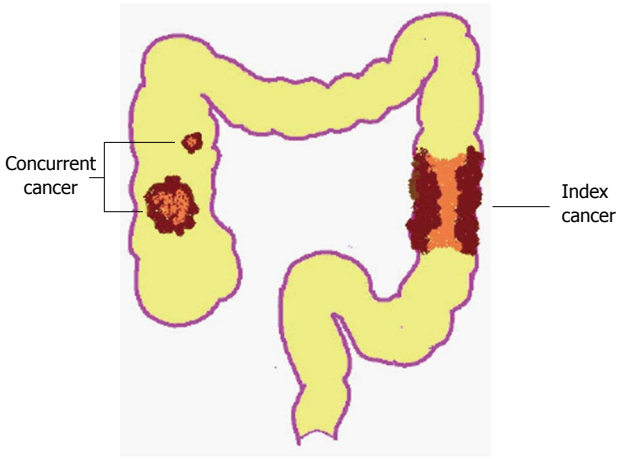

Figure 1 Illustrated model of synchronous colorectal cancers.

determination of positive or negative CT findings depended on whether both the index cancer and the concurrent cancers were simultaneously detected.

Overall sensitivity, specificity, positive predictive value, and negative predictive value for CT were calculated using cross-tabulation statistics. Fisher's exact test or χ^2 test was applied to categorical variables. Numerical data (mean maximum dimension and average distance of concurrent lesion to index tumor) are expressed as mean ± SD, and were compared using Student's *t* test. A *P* value < 0.05 was considered statistically significant.

RESULTS

A total of 374 CRC patients underwent incomplete colonoscopy and four cases of SCRCs were missed due to poor bowel preparation. A total of 1749 patients received complete endoscopy and no case of SCRCs was missed. Cancer specimens were obtained from all 2123 patients either by surgery (*n* = 1916) or colonoscopy (*n* = 207). Using CT, 56 cases of SCRCs were identified, and 36 cases were not. Regarding the missed cases, 30 patients had SCRCs detected during surgery, while the remaining six were identified during postoperative colonoscopy that was performed up to 6 mo after surgery. There were no patients with local recurrence in the anastomosis. In addition, all of the cases of SCRCs in this cohort only had one concurrent lesion detected, and none of the patients had three or more CRCs simultaneously identified. There were no significant differences in patient sex, age, gross tumor appearance, cancer differentiation, location, or the presence of colonic obstruction for the incomplete and complete colonoscopy groups (Table 1).

Size of the concurrent cancers

The mean maximum dimension of the concurrent cancers identified in the CT-negative cases was shorter than that of the CT-positive cases, and this was independent of whether a complete or incomplete colonoscopy was performed (Table 1). Regarding the missed lesions for the two groups, the largest diameters were 65 mm and 45 mm for the incomplete and complete colonoscopy groups, respectively. Moreover, the smallest cancers visualized by CT for the two groups had mean maximum dimensions of 25 mm and 20 mm, respectively, and in both groups they presented as local thickening of the colon.

Topographical relationship of the index and concurrent cancers

Concurrent cancers were located in the same bowel segment as the index cancers in 14 patients in the incomplete group and 25 in the complete group. Furthermore, > 60% of these concurrent cancers were missed by CT (Figure 3), with 68.8% (11/16) being in the incomplete colonoscopy group and 60.0% (12/20) in the complete colonoscopy group (Table 1). In contrast, 23.1% and 30.2% of cases, respectively, involved the index and concurrent cancers in different bowel segments (Table 1). Furthermore, the mean distance from index cancer to concurrent cancers was 36.77 cm (CT positive) *vs* 19.88 cm (CT negative) in the incomplete group and 36.65 cm (CT positive) *vs* 24.00 cm (CT negative) in the complete group. Therefore, topographical adjacency between index and concurrent cancers is one of the risk factors for missing diagnosis of SCRCs. The anatomical distribution of the index and concurrent cancers detected are listed in Table 2.

Sensitivity and specificity

A histological examination revealed 92 cases of SCRCs in this cohort, with 29 in the incomplete group and 63 in the complete group. The corresponding sensitivity and specificity of CT for the incomplete group was 44.8% and 93.6%, respectively (Table 3), while the positive and negative predictive values were 23.6% and 95.0%, respectively. In contrast, these values for the complete colonoscopy group were 68.3%, 97.0%, 22.2% and 98.7%, respectively.

DISCUSSION

The prevalence of SCRCs has been reported to range from 3% to 6%[3,6,7]. In the present study, the prevalence was 4.3% for 92 cases of SCRCs recorded within 10 years in a single-center database. Although the prevalence of SCRCs is not high, missed diagnosis can lead to errors in treatment and poor prognosis. Currently, colonoscopy is regarded as the best method for the direct detection of SCRCs. However, for cases that involve elongation of the colon[8], poor bowel preparation, diverticulitis, or bowel obstruction[9], colonoscopy may not be able to visualize entire segments of the colon. In a recent study, CTC was shown to be a valuable technique for preoperative colonic visualization of patients who had undergone incomplete

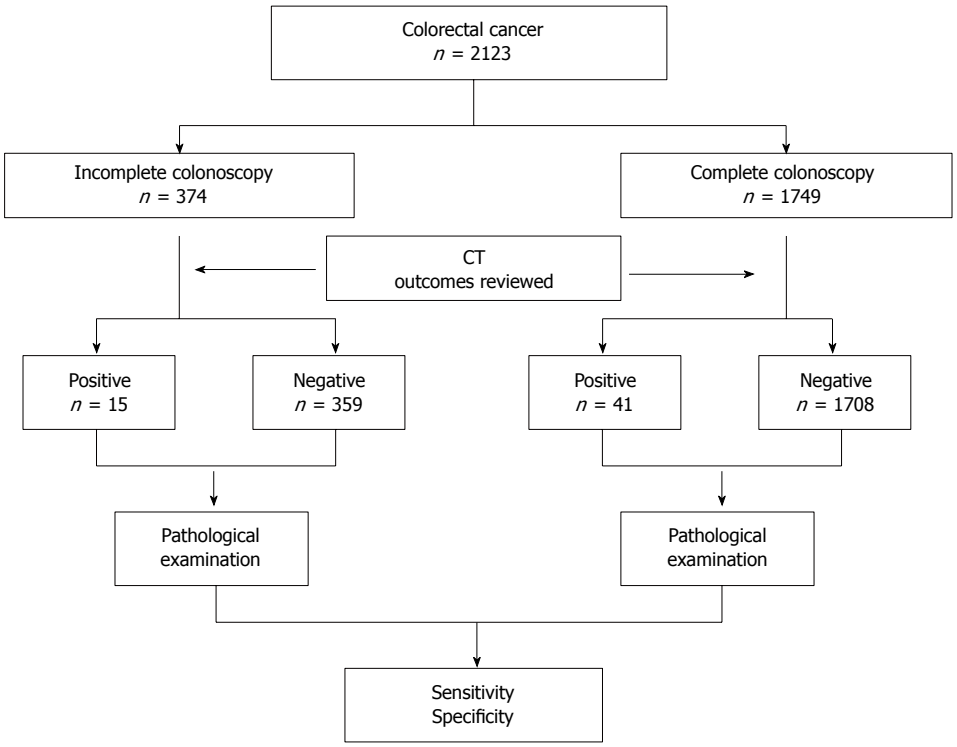

Figure 2 Flow diagram of the present study. "Positive" refers to synchronous colorectal cancers (SCRCs) detected by computed tomography (CT), while "negative" refers to SCRCs not detected by CT.

Table 1 Clinical and pathological features of the concurrent carcinomas detected by computed tomography

Concurrent lesions	Incomplete colonoscopy		*P* value	Complete colonoscopy		*P* value
	CT+ (*n* = 13)	CT- (*n* = 16)		CT+ (*n* = 43)	CT- (*n* = 20)	
Sex (M/F)	8/5	11/5		26/17	16/4	
Median age (yr)	63	69		66	63	
Mean maximum dimension (mm)	38 ± 11	24 ± 5	0.02	37 ± 13	25 ± 9	< 0.01
Gross appearance			0.20			0.18
Protruding	9	7		23	10	
Ulcerated	2	5		9	8	
Sclerotic	2	4		11	2	
Differentiation			0.17			0.06
Well	3	4		9	8	
Moderate	5	7		22	4	
Poor	5	5		12	8	
Location			0.71			0.41
Ascending colon	5	4		14	6	
Transverse colon	2	2		6	4	
Descending colon	4	5		13	6	
Sigmoid colon	2	3		8	1	
Rectum	0	2		2	3	
Colonic obstruction			0.46			-
Yes	6	10		0	0	
No	7	6		43	20	
Average distance to index tumor (cm)	36.77 ± 6.86	19.88 ± 4.16	0.04	36.65 ± 3.55	24.00 ± 5.11	0.05
Located in the same bowl segments with index tumor			0.03			0.03
Yes	3	11		13	12	
No	10	5		30	8	

colonoscopy[10,11]. Furthermore, positron emission tomography (PET)/CT has also been recommended as a supplementary technique to increase the accuracy of CTC[12]. However, the use of CTC is not widespread, mainly due to the lack of accepted guidelines for analysis of the results, and limited numbers of professionals with this experience[13]. In addition, most patients in developing countries cannot afford to undergo PET. Therefore, CT is a more accessible method, and the aim of the present study was to

Table 2 Anatomical distribution of index and concurrent cancers in this cohort

Index lesion	Concurrent lesions				
	Ascending colon	Transverse colon	Descending colon	Sigmoid colon	Rectum
Incomplete group					
Ascending colon	6	1	3	1	1
Transverse colon	0	2	1	0	0
Descending colon	2	1	4	1	0
Sigmoid colon	1	0	1	2	1
Rectum	0	0	0	1	0
Total	9	4	9	5	2
Complete group					
Ascending colon	10	1	4	3	1
Transverse colon	3	3	2	0	1
Descending colon		2	7	1	2
Sigmoid colon	0	2	1	4	0
Rectum	2	2	5	1	1
Total	20	10	19	9	5

Table 3 Overall sensitivity and specificity in the two groups

CT findings	Histological findings			Sensitivity (%)	Specificity (%)
	Positive	Negative	Total		
Incomplete group				44.8 (26.5, 64.3)	93.6 (90.5, 96.0)
Positive	13	22	35		
Negative	16	323	339		
Complete group				68.3 (55.3, 79.4)	97.0 (96.0, 97.8)
Positive	43	51	94		
Negative	20	1635	1655		
Total	92	2031	2123		

Values in parentheses are 95%CI. CT: Computed tomography.

evaluate the predictive value of CT for cases of SCRCs, especially when colonoscopy was incomplete.

The overall sensitivity of CT in the present study was 44.8% for cases involving incomplete colonoscopy and 68.8% for complete colonoscopy. Both of these percentages are lower than those reported for solitary cancers detected by CT (range: 72%-84%)[14,15]. Moreover, the sensitivity of CT for colorectal neoplasms depends on the condition of the lumen and the size of the lesion. Correspondingly, sensitivity of CT can reach as high as 95% when the lumen is distended with air, yet can be as low as 68% when the colon is collapsed[15]. In the present series, > 50% of the incomplete colonoscopy cases were due to poor bowel preparation as a result of obstruction. In addition, concurrent lesions that were smaller in size were more likely to be neglected by CT in both the incomplete and complete colonoscopy groups. For example, the average maximum dimension in the CT-positive group was about 38 mm, and it was about 25 mm in the negative group. In most cases, smaller concurrent lesions also initially presented as local thickening on CT, and in the incomplete group were often obscured by remnant fecal material caused by poor bowel preparation. However, recent studies[16-18] have demonstrated that 7%-11% of cases involving colonic wall thickening detected by CT were predicted to have an underlying carcinoma, and colonoscopy was proposed. Taken together, these results indicate that missed diagnosis occurs in a certain proportion of SCRCs when complete colonoscopy is not additionally performed.

Another important factor associated with false-negative findings on CT in the present cohort was proximity of index and concurrent cancers within the same bowel segment. Previously, the right and left colon have been reported to be frequent sites of SCRCs[4]. Moreover, the topographical proximity of carcinomas on CT may lead to misdiagnosis of SCRCs as a solitary cancer (Figure 3). For example, two individual cancers in the same bowel segment could be misinterpreted as an extension of a single cancer by CT. Generally, the direction of CRC infiltration is not along the long axis of the bowel, but rather is circumferential. Therefore, when an abnormal length of colon involvement by cancer presents on CT, SCRCs should be considered. For these suspected cases, careful palpation should be performed at the beginning of an operation because the resection range of bowel segments depends on the location of the cancers. Furthermore, if the SCRCs can be detected initially, then an extended bowel segment procedure can be scheduled in advance. Alternatively, if the concurrent cancer is detected only after the resection of the index cancer, removal of an additional bowel segment may be needed. Although supplementary resection is not complex, it is inconsistent with the *en bloc* principle of radical cancer resection.

There were some limitations associated with the present study. First, this was a retrospective single-center study. Therefore, differences in experience and professionalism among the radiologists of this center may have influenced the detection accuracy of SCRCs by CT. Second, the CT instrument used in this study was older and may have had a lower detection rate for SCRCs. Third, the operator-dependent changes should be taken into consideration, which may also have influenced the imaging results. Therefore, the results of the present study need to be verified in a multicenter prospective investigation with a large number of cases that can be analyzed using up-to-date CT instruments.

In conclusion, the present study provides evidence regarding the capacity for CT to diagnose SCRCs, especially in patients that have undergone incomplete colonoscopy. The limited detection rate of CT that was observed may have been due to several factors such as small tumor size, lumen conditions, and tumor location. However, CTC and PET/CT are not readily available at all institutions, especially in developing countries, therefore, CT remains a feasible option when complete colonoscopy cannot be performed.

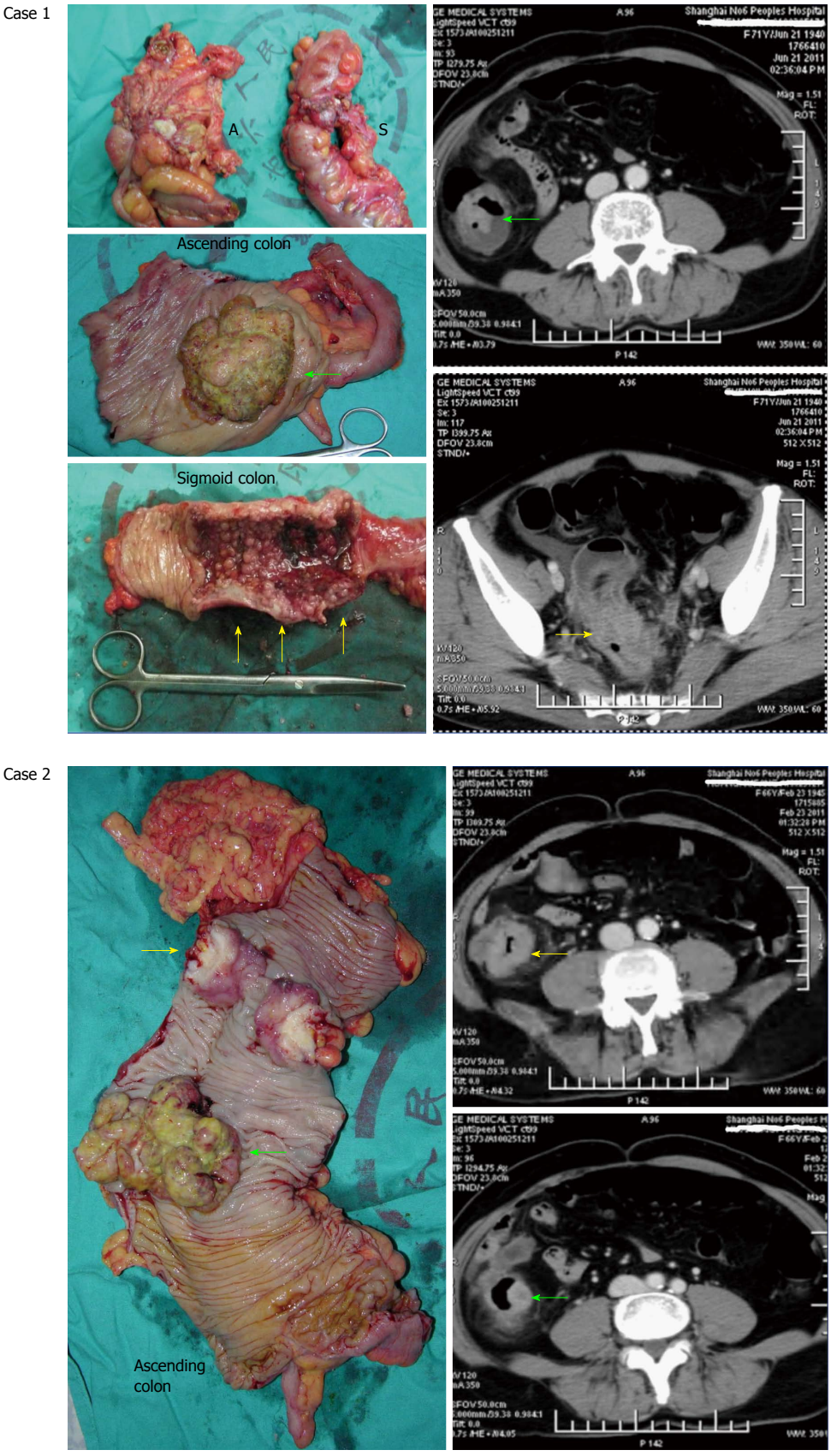

Figure 3 More than 60% of these concurrent cancers were missed by computed tomography. Case 1: A 56-year-old woman with both index and concurrent cancers located in different bowel segments that were detected by computed tomography (CT). Colonoscopy was hindered by the presence of sigmoid cancer. "A" shows the ascending colon and "S" is the sigmoid colon. The green arrows indicate the concurrent cancer and the yellow arrows indicate the index cancer; Case 2: A 68-year-old man with an index and concurrent cancer both located in the ascending colon. This case was misdiagnosed by CT as a solitary cancer due to the proximity of the two lesions. In addition, colonoscopy was hindered by the presence of a scirrhous carcinoma (index cancer indicated with yellow arrows). The green arrows indicate the concurrent cancer.

Accordingly, it is important to identify the factors that can lead to false-negative results. Furthermore, both careful exploration during intraoperative colonoscopy[19,20] and close monitoring during the follow-up period are key to obtaining accurate diagnosis and treatment of SCRCs.

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COMMENTS

Background

Synchronous colorectal cancers (SCRCs) refer to two or more primary colorectal cancers (CRCs) in a single individual with a prevalence ranging from 3% to 6%. SCRCs are clinically important because neglect of lesions leads to remnant cancer. Colonoscopy is ideal for detecting synchronous lesions but some factors may prevent the colonoscope from visualizing whole bowel segments. Conventional computed tomography (CT) is usually applied in these cases to examine the remnant bowel not accessible to colonoscopy.

Research frontiers

CT colonography (CTC) is also known as virtual colonoscopy. With advancements made in scanning technology and three-dimensional (3D) postprocessing modalities, this method has been used to detect colorectal cancers, especially small tumors. CTC has been shown to be a valuable technique for preoperative colonic visualization of patients who have undergone incomplete colonoscopy. However, good bowel preparation is necessary for accurate diagnosis.

Innovations and breakthroughs

Currently, colonoscopy is regarded as the best method for the direct detection of SCRCs. However, for cases that involve elongation of the colon, poor bowel preparation, diverticulitis, or bowel obstruction, colonoscopy may not be able to visualize entire segments of the colon. CTC or positron emission tomography (PET) was shown to be valuable for preoperative detection of lesions in patients undergoing incomplete colonoscopy. PET and CTC in most patients in developing countries are not widespread, mainly due to limited numbers of well-trained professionals, and high expense. Therefore, CT is a more accessible method, and the present study was the first to evaluate the predictive value of CT for cases of SCRCs, especially when colonoscopy was incomplete.

Applications

The study suggested that CT remains a feasible option when complete colonoscopy cannot be performed. However, the sensitivity is limited when the tumor is small or index cancer is adjacent to concurrent cancers.

Terminology

SCRCs refer to two or more primary CRCs consistent with the following key elements: (1) each tumor must be proved to be malignant; (2) each tumor must be distinct; (3) the probability of one tumor being a metastasis of another must be excluded; and (4) the synchronous lesions must be diagnosed simultaneously or within 6 mo of initial diagnosis.

Peer-review

Pang *et al* evaluated the diagnostic accuracy of CT in patients with SCRCs. It was a well-designed study, and the paper is well written. It addresses an interesting clinical area.

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ORIGINAL ARTICLE

Retrospective Study

Prognostic factors and survival in patients with gastric stump cancer

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Abstract

AIM: To elucidate the clinicopathological characteristics and prognostic factors of gastric stump cancer (GSC).

METHODS: The clinical data for 92 patients with GSC were collected at Fudan University Shanghai Cancer Center. The prognostic factors were analyzed with Cox proportional hazard models.

RESULTS: GSC tended to occur within 25 years following the primary surgery, when the initial disease is benign, whereas it primarily occurred within the first 15 years post-operation for gastric cancer. Patients with regular follow-up after primary surgery had a better survival rate. The multivariate Cox regression analysis revealed that Borrmann type Ⅰ/Ⅱ (HR = 3.165, 95%CI: 1.055-9.500, *P* = 0.040) and radical resection (HR = 1.780, 95%CI: 1.061-2.987, *P* = 0.029) were independent prognostic factors for GSC. The overall 1-, 3-, and 5-year survival rates of the 92 patients were 78.3%, 45.6% and 27.6%, respectively. The 1-, 3-, and 5-year survival rates of those undergoing radical resection were 79.3%, 52.2%, and 37.8%, respectively. The 5-year survival rates for stages Ⅰ, Ⅱ, Ⅲ, and Ⅳ were 85.7%, 47.4%, 16.0%, and 13.3%, respectively (*P* = 0.005).

CONCLUSION: The appearance of GSC occurs sooner in patients with primary malignant cancer than in patients with a primary benign disease. Therefore, close follow-up is necessary. The overall survival of patients with GSC is poor, and curative resection can improve their prognosis.

Key words: Gastric stump cancer; Clinicopathological characteristics; Prognosis

Core tip: We retrospectively analyzed 92 patients. This study indicated that gastric stump cancer (GSC) has unique clinicopathologic characteristics, early detection of GSC is indeed possible, close follow-up is necessary and the radical resection may significantly improve the survival.

Huang H, Wang W, Chen Z, Jin JJ, Long ZW, Cai H, Liu XW, Zhou Y, Wang YN. Prognostic factors and survival in patients with gastric stump cancer. *World J Gastroenterol* 2015; 21(6): 1865-1871 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i6/1865.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i6.1865

INTRODUCTION

The concept of gastric stump cancer (GSC) was originally described in the 1920s[1] , and was defined as a carcinoma occurring in the gastric remnant at least 5 years post-surgery for benign peptic ulcer disease. Currently, the concept of GSC has been expanded to include recurrence after gastric cancer resection[2,3].

Gastric stump as a precancerous condition remains a substantial clinical concern. The incidence of GSC accounts for 1%-7% of all gastric carcinomas following gastrectomy, and this frequency continues to increase[3-5]. Nevertheless, GSC is often described as a tumor with poor prognosis, and poor curative resection rates (38%-40%)[6,7]. The 5-year survival rate is only 7%-20% because GSC has unique biological features compared with conventional stomach cancer, and GSC is commonly diagnosed at an advanced stage[4,8,9].

The present study aimed to clarify the clinicopathological characteristics and operative methods for patients with GSC in order to improve their long-term outcomes.

MATERIALS AND METHODS

Patients

We retrospectively analyzed 92 patients who had undergone stomach resection between January 2003 and December 2012 at the Gastric Surgery Department of Fudan University Shanghai Cancer Center, China. All the patients were diagnosed through barium meal, endoscopic, and pathological examinations. Patient information was obtained from the medical records of Fudan University Shanghai Cancer Center. The clinical symptoms included abdominal pain, emesis, dysphagia, weight loss, anemia, and weakness. The clinical variables included age, gender, initial gastric disease, interval from initial surgery, reconstruction type of the first operation, type of gastric resection, tumor location, and tumor stage.

Follow-up

All patients were regularly contacted by telephone, and all patients received a follow-up. The duration of the follow-up period was defined as the interval from treatment date to the date of death or the last follow-up. The last follow-up occurred on March 31, 2014. Sixty-seven patients died at the last follow-up.

Evaluation

Tumor-node-metastasis (TNM) classification of gastric carcinoma was based on the seventh edition of the American Joint Committee on Cancer staging system. The surgical and pathological findings were recorded according to the Japanese Classification of Gastric Carcinoma, and the histological types were classified as differentiated or undifferentiated. The differentiated type included papillary adenocarcinoma and well/moderately differentiated adenocarcinoma, while the undifferentiated type included poorly or undifferentiated adenocarcinoma, signet ring cell carcinoma, and mucinous carcinoma. To calculate the survival curves, only patients who underwent tumor resection were included because these were the only patients with complete histopathological data and staging data.

Statistical analysis

Statistical analyses were performed using the SPSS version 19.0 statistical software package (SPSS IBM, United States). All continuous variables are presented as the median (range). The cumulative cause-specific overall survival rates were calculated using the Kaplan-Meier method. The log-rank test was used to assess differences between clinical factors. Cox proportional hazard models were used to determine which clinicopathological variables were predictive of GSC. $P < 0.05$ was regarded as significant.

RESULTS

The median age was 60.8 (range: 37-91) years, and the male:female ratio was 4.4:1. The median interval time from the initial operation to the development of GSC was 16.5 years (range: 1-40 years). The latency periods were different between benign disease and gastric cancer. GSC tended to occur within 25 years post-operation with a benign initial disease and within the first 15 years post-operation for gastric cancer (Table 1, Figure 1). Seventy-six (82.6%) patients had clinical symptoms, including abdominal pain, emesis, dysphagia, weight loss, anemia and weakness. Other patients were diagnosed *via* routine endoscopy.

The detailed clinicopathological characteristics of all the patients are listed in Table 1. Concerning the initial gastric disease, 38 (41%) patients had benign disease, and 54 (59%) patients had gastric cancer. In total, 26 (28%) patients underwent Billroth-Ⅰ reconstruction, and 65 (71%) patients received Billroth-Ⅱ

Table 1 Clinicopathologic features of the 92 patients with gastric stump cancer *n* (%)

Characteristic	Value
Age (yr)[1]	60.8 ± 10.0
Sex	
Male	75 (81)
Female	17 (19)
Initial gastric disease	
Benign	38 (41)
Malignant	54 (59)
Interval time from initial surgery (yr)[1]	16.5 ± 13.0
Regular follow-up after primary surgery	
Yes	76 (83)
No	16 (17)
Reconstruction of primary surgery	
Billroth-Ⅰ	26 (28)
Billroth-Ⅱ	65 (71)
Roux-en-Y	1 (1)
Location	
Anastomotic	59 (64)
Non-anastomotic	33 (36)
Tumor size (mm)[1]	4.0 ± 2.0
Borrmann type	
Ⅰ	3 (3)
Ⅱ	13 (14)
Ⅲ	69 (75)
Ⅳ	7 (8)
Depth of invasion	
T1	0 (0)
T2	6 (7)
T3	5 (5)
T4	81(88)
Lymph node involvement	
N0	35 (38)
⩾ N1	57 (62)
Presence of distant metastasis	
M0	63 (68)
⩾ M1	29 (32)
Stage	
Ⅰ	8 (9)
Ⅱ	25 (26)
Ⅲ	30 (33)
Ⅳ	29 (32)
Histology	
Differentiated	18 (20)
Undifferentiated	74 (80)
Type of treatment	
Curative resection	58 (63)
Palliative resection	12 (13)
Chemotherapy/radiotherapy	16 (17)
No treatment	6 (7)

[1]Age, tumor size and disease-free time are mean ± SD.

reconstruction. Most patients (59, 64%) had malignant lesions at the anastomotic site, and the median tumor size was 4.0 cm. Additionally, Borrmann Ⅲ (69, 75%) was the most common type based on the gross appearance. Histology revealed that the most common type of cancer was undifferentiated (80%). The TNM classification was as follows: stage Ⅰ in 8 patients (9%), stage Ⅱ in 25 (26%), stage Ⅲ in 30 (33%), and stage Ⅳ in 29 (32%). The lymph node metastasis rate in patients with GSC was 38% (35/92).

Seventy patients received surgical treatments, and 58 patients were qualified for radical surgery. The resectability rate was 59.8%, and 12 patients underwent palliative resection. Twenty-two patients declined surgery. Among them, 6 patients declined treatment, and 16 patients chose to receive chemotherapy/radiotherapy.

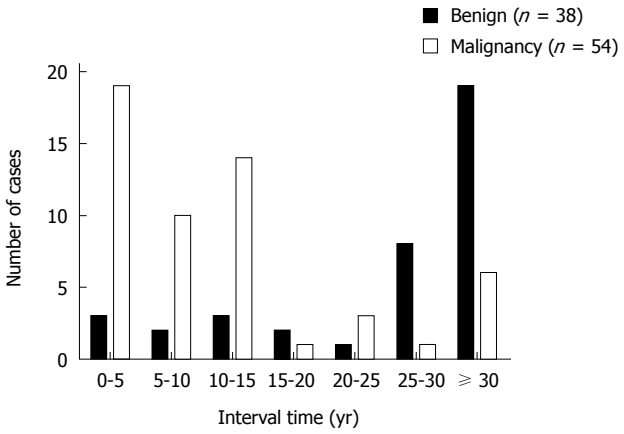

Figure 1 Time trend of stump carcinoma every 5 years for the primary gastric disease.

All 92 patients provided complete follow-up data. At the time of the last follow-up, 67 (72.8%) patients had died, and 25 (27.2%) patients were alive. The median survival duration was 29 mo, and the cumulative 1-, 3-, and 5-year overall survival rates were 78.3%, 45.6%, and 27.6%, respectively (Figure 2A). The survival curves also suggested that patients with regular follow-up after primary surgery had a better survival rate (Figure 2B and C). Their 1-, 3-, and 5-year survival rates were 93.8%, 75.0%, and 66.7%. However, the 1-, 3-, and 5-year survival rates were 87.5%, 47.7%, and 25.6%, respectively, for patients without regular follow-up after the primary surgery. Figure 2D demonstrates that patients with Borrmann type Ⅱ cancer had the best survival rate (P = 0.0083). The 1-, 3-, and 5-year survival rates of the 58 patients who underwent radical resection were 79.3%, 52.2%, and 37.8%, respectively. Among them, 3 (3.3%) patients died during the peri-operative period. The peri-operative mortality was similar to that of patients with conventional gastric cancer (2%-3%)[10]. The 1-, 3-, and 5-year survival rates of the 12 patients who received palliative resection were 66.7%, 25.0%, and 0%, respectively, and there were significant differences between the three groups (P = 0.0007) (Figure 2E). Table 2 shows the group characteristics according to the tumor stage. The 5-year survival rates for stages Ⅰ, Ⅱ, Ⅲ, and Ⅳ were 85.7%, 47.4%, 16.0%, and 13.3%, respectively (P = 0.005).

DISCUSSION

Despite a decline in the overall incidence of gastric cancer[11], the incidence rate of GSC has increased during recent decades[3-5]. The incidence of GSC accounts for 1%-2% of all gastric cancers in Japan[2].

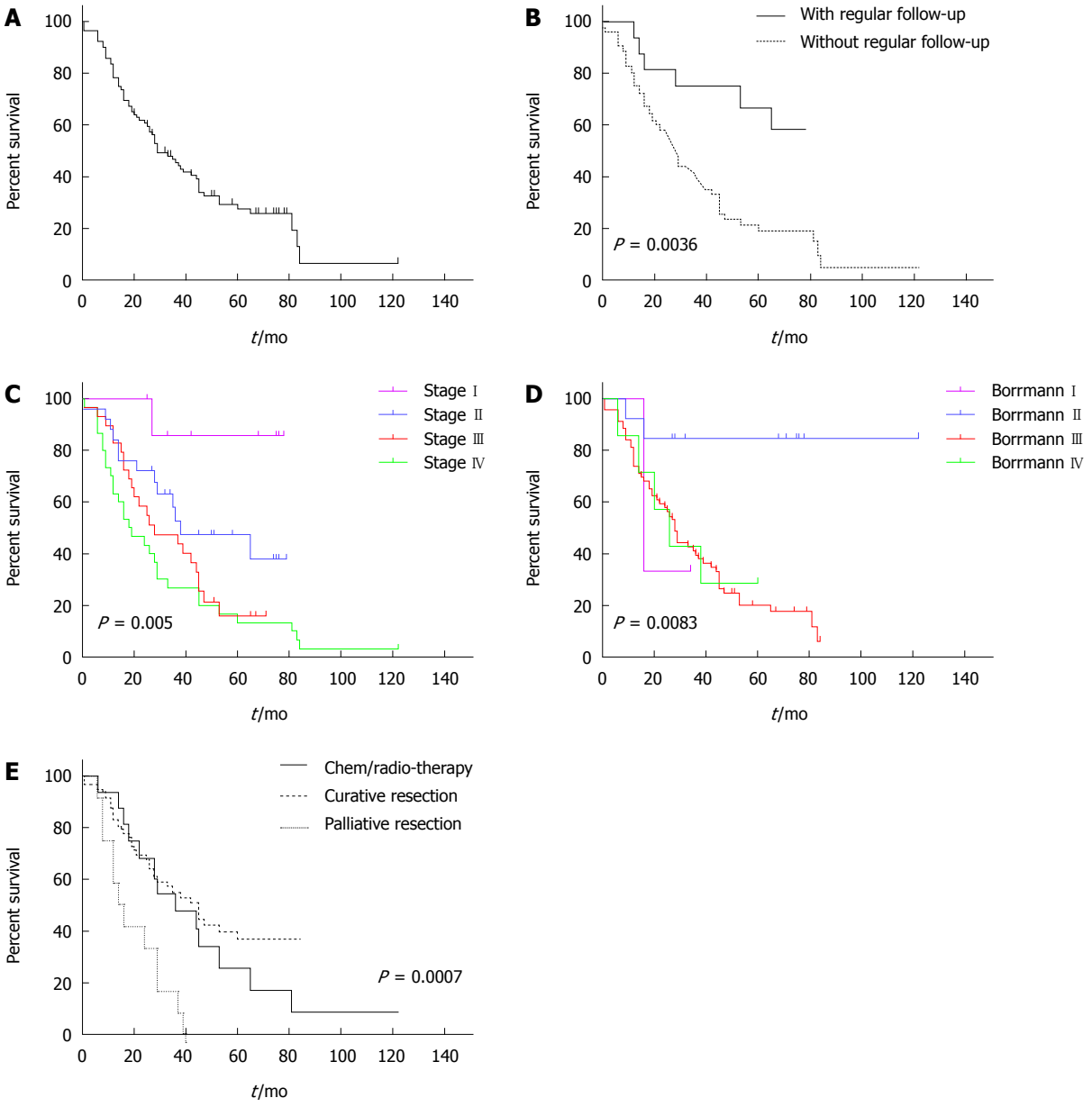

Figure 2 Overall survival proportions. A: Overall survival proportions of 92 patients with gastric stump cancer; B: Overall survival proportions between patients with and without regular follow-up after primary surgery are significantly different. The patients without regular follow-up after primary surgery had significantly poorer overall survival; C: Overall survival proportions for patients with different stages; D: Overall survival proportions for patients with different Borrmann type; E: Overall survival proportions in gastric stump cancer according to type of treatments.

This increase indicates that we will face increasing challenges in the future.

To the best of our knowledge, the type of initial gastric disease has significant impact on the latency to GSC. The median interval for patients with benign disease is 30 years, and that for patients with gastric cancer is 12 years[12]. The incidence of GSC after gastric resection increases over time[13]. In the present study, the interval for benign disease was significantly longer than that for gastric cancer. Additionally, we observed different interval periods between benign disease and gastric cancer (Figure 1). The median interval time from initial operation to the development of GSC was 16.5 years (range: 1-40 years), which is consistent with other studies[9]. Figure 1 indicates that GSC tends to occur within 25 years following the initial benign disease, and during the 15 years post-operation for patients who have gastric cancer.

The clinical symptoms of GSC have no obvious specificity, and GSC usually manifests as upper abdominal pain, loss of appetite, swallowing difficulty, vomiting, weight loss, and anemia. GSC is often diagnosed at an advanced stage due to the lack of early symptoms. It is easily misdiagnosed as an ulcer recurrence or anastomotic inflammation, which can lead to a delayed diagnosis and the loss of the best treatment opportunity. The remnant stomach is viewed as a precancerous lesion. The specific etiology of GSC is currently

Table 2 Comparison of baseline characteristics according to tumor stage *n* (%)

Variable		Ⅰ (*n* = 8)	Ⅱ (*n* = 25)	Ⅲ (*n* = 30)	Ⅳ (*n* = 29)
Histology	Differentiated	4 (4.3)	6 (6.5)	6 (6.5)	2 (2.2)
	Undifferentiated	4 (4.3)	19 (20.6)	24 (26.1)	27 (29.3)
Tumor Location	Anastomotic	4 (4.3)	17 (18.5)	21 (22.8)	17 (18.5)
	Non-anastomotic	4 (4.3)	8 (8.7)	9 (9.8)	12 (13.0)
Initial gastric disease	Benign disease	4 (4.3)	8 (8.7)	13 (14.1)	13 (14.1)
	Gastric cancer	9 (9.8)	17 (18.5)	17 (18.5)	16 (17.4)

Values in parentheses are percentages.

Table 3 Univariate and multivariate survival analyses in gastric stump cancer patients

Variable	Univariate[1]	Multivariate[2]		
	P value	HR	95%CI	*P* value
Age (yr) (≥ 65 *vs* < 65)	0.896			
Sex ratio (male *vs* female)	0.302			
Initial gastric disease (benign *vs* cancer)	0.443			
Interval time from initial surgery (yr) (≥ 15 *vs* < 15)	0.428			
Reconstruction of first operation (B-Ⅰ *vs* B-Ⅱ)	0.357			
Regular follow-up (yes *vs* no)	0.004	1.332	0.539-3.287	0.535
Tumor size (mm) (≥ 40 *vs* < 40)	0.040	0.707	0.415-1.206	0.203
Location (anastomotic *vs* non-anastomotic)	0.857			
Histology(differentiated *vs* undifferentiated)	0.005	0.470	0.212-1.038	0.062
Borrmann type (Ⅰ-Ⅱ *vs* Ⅲ-Ⅳ)	0.001	3.165	1.055-9.500	0.040
Depth of invasion (T1-T2-T3 *vs* T4)	0.305			
Lymph node involvement (NO *vs* ≥ N1)	0.254			
Presence of distant metastasis (M0 *vs* M1)	0.004			
Stage (Ⅰ-Ⅱ *vs* Ⅲ-Ⅳ)	0.002	0.603	0.323-1.124	0.111
Type of treatment (curative *vs* other treatments)	0.001	1.780	1.061-2.987	0.029

[1]The Kaplan-Meier method, and significance was determined by the log-rank test; [2]Multivariate survival analysis was performed using Cox proportional hazard models.

unclear. According to our data, 16 (17%) patients had no clinical symptoms, and the malignant lesions of the remnant stomach were identified through periodic endoscopies. Most of these patients had early-stage GSC, and their 5-year survival rate was 66.7%. The 5-year survival rate of patients who had clinical symptoms was 19.0%. The survival curves also suggested that patients who were followed had better survival rates (Figure 2B). Regular follow-up can facilitate early detection and early therapy, which improve the survival rate.

Previous studies report the unique clinicopathological characteristics for GSC[2,8,14]. Our analysis demonstrated that the histological type was associated with prognosis (Table 3). The prognosis of patients with GSC was ultimately determined by the Borrmann lesion type and radical resection. Tumor location was also an important factor for predicting surgical outcomes[15-17]. The tumors commonly developed at the site of the gastrojejunal anastomosis. Some scholars thought that the type of reconstruction was associated with GSC[18-20]. In our study the Billroth-Ⅱ reconstruction and anastomotic groups were more likely to develop GSC, although there was no significant difference due to the small number of patients.

Some drugs have been used to treat GSC in Japan[21]. However, in our study, surgery was still the most effective treatment. Although there were no significant differences in the histopathologic categories and tumor location of GSC compared with primary proximal gastric cancer[22-24], GSC is the most frequently occurring tumor after the initial surgery and occurs in the remnant stomach due to an abnormal anatomy. Additionally, because surgery for GSC is the second surgery, there is an increased number of adhesions around the residual stomach. Therefore, radical surgery is more difficult than ordinary surgery. There were no severe complications during and after our operations, therefore, radical surgery is feasible. The survival rate in the radical resection group was significantly higher than those in the palliative resection group and the chemotherapy/radiotherapy group. Our results demonstrated that chemotherapy/radiotherapy has a good short-term curative effect, but the long-term curative effect is still poor. Previous studies have reported higher incidences of postoperative complications in patients with GSC than in patients with primary gastric cancer[25]. Radical resection is still the best treatment option and may improve the survival outcomes of patients with GSC.

Admittedly, our study had a relatively small sample and was based on a retrospective analysis. However, even after acknowledging these limitations, we can

draw some meaningful conclusions regarding GSC.

In conclusion, the findings in the present study led us to draw the following conclusions. Borrmann type Ⅰ/Ⅱ and radical resection are independent prognostic factors for patients with GSC. Early detection of GSC is possible. Regular endoscopies and gastric biopsies for subtotal gastrectomy patients have significant impact on postoperative survival. Therefore, it is necessary and feasible to perform repeated endoscopic follow-ups. If GSC is diagnosed, surgery should be performed. Additionally, radical resection may significantly improve the long-term survival of patients.

COMMENTS

Background

Gastric stump as a precancerous condition remains a substantial clinical concern. However, few studies explored the clinicopathological characteristics and prognostic factors for gastric stump cancer (GSC).

Research frontiers

GSC is often described as a tumor with a poor prognosis because of the unique biological features and it is commonly found at an advantage stage. Therefore, it is necessary to explore the clinicopathological characteristics in a great number of clinical cases.

Innovations and breakthroughs

This is the first comprehensive clinical trial about GSC in China. We observed the unique clinicopathological characteristics and prognostic factors for GSC, and the radical resection may significantly improve the long-term survival.

Applications

The results of the present study suggest that regular follow-up by endoscopy and gastric biopsy is necessary for subtotal gastrectomy patients, and the radical resection may significantly improve the long-term survival.

Terminology

The incidence of GSC accounts for 1%-7% of all gastric carcinomas following gastrectomy, and this frequency continues to increase. Gastric stump as a precancerous condition remains a substantial clinical concern. GSC is often described as a tumor with poor prognosis.

Peer-review

The manuscript is a remarkable article regarding GSC, which remains a substantial clinical concern and has its unique clinicopathological characteristics, as the appearance of GSC is earlier in the primary malignant cases than in the primary benign cases. So it is necessary to follow patients closely.

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ORIGINAL ARTICLE

Retrospective Study

Application of air insufflation to prevent clinical pancreatic fistula after pancreaticoduodenectomy

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Abstract

AIM: To introduce an air insufflation procedure and to investigate the effectiveness of air insufflation in preventing pancreatic fistula (PF).

METHODS: From March 2010 to August 2013, a total of 185 patients underwent pancreaticoduodenectomy (PD) at our institution, and 74 patients were not involved in this study for various reasons. The clinical outcomes of 111 patients were retrospectively analyzed. The air insufflation test was performed in 46 patients to investigate the efficacy of the pancreaticojejunal anastomosis during surgery, and 65 patients who did not receive the air insufflation test served as controls. Preoperative assessments and intraoperative outcomes were compared between the 2 groups. Univariate and multivariate analyses were performed to identify the risk factors for PF.

RESULTS: The two patient groups had similar baseline demographics, preoperative assessments, operative factors, pancreatic factors and pathological results. The overall mortality, morbidity, and PF rates were 1.8%, 48.6%, and 26.1%, respectively. No significant differences were observed in either morbidity or mortality between the two groups. The rate of clinical PF (grade B and grade C PF) was significantly lower in the air insufflation test group, compared with the non-air insufflation test group (6.5% *vs* 23.1%, $P = 0.02$). Univariate analysis identified the following parameters as risk factors related to clinical PF: estimated blood loss; pancreatic duct diameter $\leqslant$ 3 mm; invagination anastomosis technique; and not undergoing air insufflation test. By further analyzing these variables with multivariate logistic regression, estimated blood loss, pancreatic duct diameter $\leqslant$ 3 mm and not undergoing air insufflation test were demonstrated to be independent risk factors.

CONCLUSION: Performing an air insufflation test could significantly reduce the occurrence of clinical PF after PD. Not performing an air insufflation test was an independent risk factor for clinical PF.

Key words: Pancreatic fistula; Pancreaticoduodenectomy; Air insufflation test; Surgery; Morbidity

Core tip: The present study introduces the application of the air insufflation test for the prevention of pancreatic fistula (PF) and investigates its effectiveness. This clinical study confirms that the air insufflation test can significantly reduce the occurrence of clinical PF. In addition, estimated blood loss, pancreatic duct diameter $\leqslant$ 3 mm and not performing an air insufflation test are independent risk factors for clinical PF.

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INTRODUCTION

Pancreaticoduodenectomy (PD) is the standard operation for the resection of periampullary diseases. In recent years, the mortality rate has decreased dramatically to less than 5% in high-volume centers due to improved intraoperative management and better postoperative care. Unfortunately, there has not been a similar reduction in the pancreatic fistula (PF) rate, which has remained at approximately 30%[1-3].

Pancreatic leakage has been the primary factor linked with death in some case studies[4]. Various risk factors related to PF have been identified, such as a main pancreatic duct diameter of 3 mm or less[2,5-8], soft pancreatic parenchyma[7,9-12] and intraoperative blood loss[2,11-13]. Other risk factors, such as heart disease[9,14], age[6], male sex[11], cirrhotic liver and body mass index[7], have also been reported. In addition, to prevent PF, numerous strategies have been applied in previous literatures. Intraoperative techniques, such as modified reconstruction with pancreaticogastrostomy[15], pancreatic duct ligation[16], the use of omentum or the falciform ligament[17,18] and obliteration of the pancreatic duct with glue[19], have been performed. Postoperative management, including the use of somatostatin analogs, has also been reported. However, none of these strategies has investigated pancreatic leakage during surgery. Furthermore, most of these strategies have been controversial[20-23] and have not been routinely used in most hospital centers. More effective and confirmative surgical techniques are needed to prevent PF.

The present study introduced the air insufflation test, a simple and effective technique, for the prevention of PF. The detailed process of performing an air insufflation test during surgery and its efficacy in preventing PF are presented.

MATERIALS AND METHODS

Protocol

PD was performed in 185 consecutive patients between March 2010 and August 2013 at the Qilu Hospital of Shandong University in China. The exclusion criteria applied to: patients undergoing emergency PD for trauma; patients with ongoing acute pancreatitis at the time of surgery; and operations performed by surgeons without a professional title. A total of 111 patients were enrolled in this study, and these patients were divided into 2 groups according to whether they received the air insufflation test during surgery or not. In total, 46 patients [the air insufflation test (AIT) group] received the air insufflation test during surgery, and 65 patients (controls) did not receive the air insufflation test during surgery.

Preoperative demographics and clinical information of the patients were retrospectively obtained from the patients' medical records (Table 1). Preoperative biliary drainage was the main preoperative invasive treatment. For patients with a total bilirubin level greater than 170 μmol/L or with poor general health conditions, preoperative biliary drainage was performed.

Surgical techniques and postoperative management

The operations were performed by surgeons with professional titles specializing in hepatopancreatobiliary surgery. Conventional or pylorus-preserving PD was performed according to the decision of the individual surgeon. Segmental resection of the portal vessels or superior mesenteric vessels was performed if a pancreatic head mass was inseparable from the vessels. To reestablish gastrointestinal continuity, a two-layer end to side pancreatic duct to jejunal mucosa anastomosis (duct to mucosa) or a two-layer end to side invagination anastomosis was performed (Figure 1). After pancreaticojejunal anastomosis, an end to side hepaticojejunostomy and an antecolic gastrojejunostomy or duodenojejunostomy were performed, using the same jejunal loop. Depending on the diameter of the pancreatic duct, a Fr 4 to 10 polyvinyl catheter with multiple perforations was inserted into the pancreatic duct.

To investigate the efficacy of the pancreaticojejunal anastomosis, the air insufflation test (Figure 2) was performed in the AIT group. An intestinal clamp was used to close the distal intestinal loop approximately 6 cm from the pancreaticojejunal anastomosis. Then, the anastomosis was submerged in irrigation fluid, and air was injected gently with a 1 or 5 mL syringe through the pancreatic duct stent to determine whether there were bubbles generated in the irrigation fluid. It must be noted that air should be gently injected into the jejunal stump to prevent the pressure in the jejunal lumen from ascending too rapidly. In addition, the pressure in the jejunal

Table 1 Baseline demographics

	AIT group (n = 46)	Non-AIT group (n = 65)	P value
Age (yr)	55.3 ± 11.5	58.1 ± 7.8	0.15
Gender			0.64
Male	24	31	
Female	22	34	
Main presenting symptom			
Jaundice	25	35	0.96
Abdominal pain	10	24	0.09
Cholangitis	3	8	0.50
ASA			0.64
Ⅱ	41	56	
Ⅲ	5	9	
Preoperative ALT	226.0 ± 204.2	193.6 ± 205.0	0.41
Preoperative AST	150.2 ± 152.1	138.3 ± 150.7	0.68
Preoperative bilirubin (μmol/L)	189.0 ± 171.9	137.11 ± 131.8	0.08
Preoperative albumin (g/L)	66.2 ± 8.5	65.8 ± 7.8	0.81
Presence of comorbid illness	23	27	0.38
Mellitus diabetes	8	4	0.06
Chronic pulmonary disease	2	4	0.68
Artery hypertension	9	13	0.96
Coronaropathy	4	6	1.00
Preoperative biliary drainage			0.60
Yes	21	33	
No	25	32	
Smoking	13	14	0.42

ASA: American Society of Anesthesiologists; ALT: Alanine aminotransferase; AST: Aspartate transaminase; AIT: Air insufflation test.

stump should be sufficiently moderate to generate bubbles but not damage the anastomosis. In clinical practice, we regarded the pressure as moderate when the tension of the jejunal wall was the same as that of normal liver tissue or the oral labia. If bubbles were present during the test, the anastomosis where the bubbles were generated was sutured. Then, the anastomosis was tested again until no bubbles were found. If the leakage could not be resolved, re-anastomosis was performed. Prophylactic drains were routinely placed posterior to the pancreaticojejunal anastomosis and the hepaticojejunal anastomosis. Fibrin glue was not used in any of the patients.

Prophylactic antibiotics and somatostatin or octreotide were administered to all of the patients for 72 h postoperatively and during the first postoperative week, respectively. The nasogastric tube was removed when bowel sounds returned. An oral diet was initiated 5 to 7 d after surgery, depending on the patient's condition. The volume and characteristics of the drainage fluid were monitored every day. Amylase levels were measured on postoperative days 1, 3, 5, and 7 and when the characteristics of the drainage fluid changed, or abdominal symptoms occurred. If there was no evidence of PF, the pancreatic duct drainage catheter was locked 10 d after surgery and removed 48 h later if no abnormalities occurred. If a PF occurred, the catheter was placed *in situ* until the leakage was resolved.

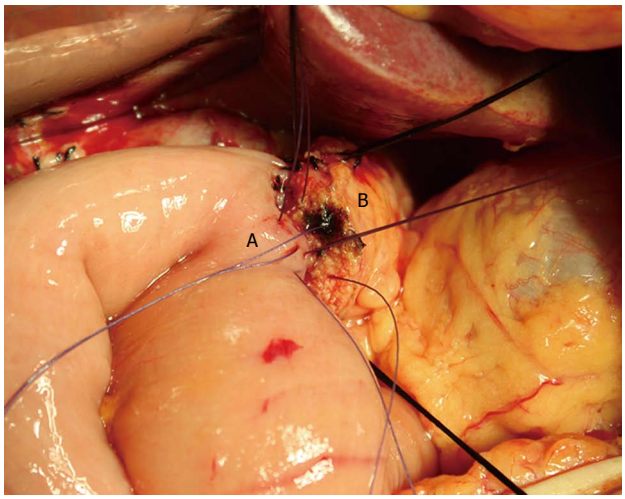

Figure 1 Pancreaticojejunal anastomosis. This picture shows a two-layer end to side pancreatic duct to jejunal mucosa anastomosis. A: Jejunum; B: Pancreas.

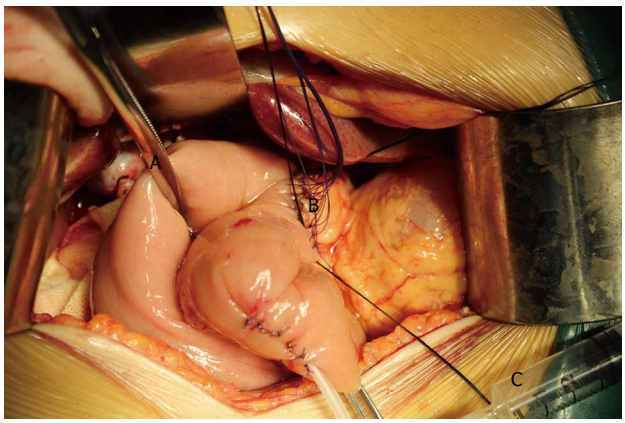

Figure 2 Air insufflation test to investigate the pancreaticojejunal anastomosis. An intestinal clamp (A) was used to close the distal intestinal loop approximately 6 cm from the pancreaticojejunal anastomosis (B). Then, the anastomosis was submerged in irrigation fluid, and air was injected gently with a 1 or 5 mL syringe (C) through the pancreatic duct stent to determine whether there were bubbles generated.

Study end points

The primary end point was PF. The secondary end points were mortality and morbidity, including delayed gastric emptying (DGE), intra-abdominal hemorrhage, bile fistula, intra-abdominal infection, intra-abdominal collection, and heart failure.

Mortality and morbidity were defined as death or complications, respectively, occurring within 30 d of surgery. PF was defined as drainage of any measurable volume of fluid on or after postoperative day 3 with an amylase content greater than 3 times the serum amylase activity. The three different grades of PF (grades A-C) were defined according to the clinical impact on the patient's hospital course[24]. Grade B and grade C PF were regarded as clinical PF. DGE represented the inability to return to a standard

Table 2 Intraoperative data and pathological diagnoses

	AIT group (n = 46)	Non-AIT group (n = 65)	P value
Operative factors			
Combined vascular resection cases	1	2	1.00
Duration of operation (min)	372.9 ± 106.5	344.7 ± 86.4	0.13
Estimated blood loss (mL)	347.3 ± 195.7	289.7 ± 159.9	0.09
Blood transfusion cases	13	20	0.78
Pancreatic factors			
Pancreatic duct diameter			0.76
⩽ 3 mm	31	42	
> 3 mm	15	23	
Pancreatic texture			0.58
Soft	34	51	
Hard	12	14	
Pancreatic anastomosis technique			0.17
Duct to mucosa anastomosis	20	20	
End to side invagination anastomosis	26	45	
Pathological diagnoses			0.66
Malignant disease	39	57	
Pancreatic carcinoma	11	17	0.79
Ampullar carcinoma	10	12	0.67
Cholangiocarcinoma	8	12	0.89
Duodenal carcinoma	10	16	0.72
Benign diseases	7	8	

Data are expressed as mean ± SD. AIT: Air insufflation test.

diet by the end of the first postoperative week and included prolonged nasogastric intubation of the patient[25]. Postoperative hemorrhage was defined in accordance with the International Study Group of Pancreatic Surgery guidelines, based on the time of onset (early or late hemorrhage), the location (intraluminal or extraluminal), and the severity (mild or severe)[26].

Statistical analysis

The statistical analyses were performed using SPSS software, version 19.0 for Windows (SPSS Inc., Chicago, IL, United States). Continuous data are expressed as mean ± SD. The comparison of continuous or categorical variables was performed with Student's t-test or the χ^2 test (or Fisher's exact test), respectively. Significant variables from the univariate analysis were subjected to multivariate stepwise logistic regression analysis. A P value ⩽ 0.05 was considered significant.

RESULTS

Clinical characteristics

The baseline demographics of the 111 patients included in the present study are shown in Table 1. These results suggested that the two groups were well matched for age, sex, main presenting symptom, American Society of Anesthesiologists physical status score, alanine aminotransferase, aspartate transaminase, preoperative bilirubin, preoperative albumin, presence of comorbid illness, preoperative biliary drainage and smoking status (Table 1).

Table 3 Postoperative outcomes

	AIT group (n = 46)	Non-AIT group (n = 65)	P value
Hospital mortality	1	1	1.00
Morbidity	20	34	0.36
Pancreatic fistula	9	20	0.19
Grade A	6	5	0.54
Grade B	2	8	0.02
Grade C	1	7	
Delayed gastric emptying	5	7	1.00
Hemorrhage	3	8	0.50
Bile fistula	3	8	0.50
Intraabdominal collection	4	10	0.30
Intraabdominal infection	3	12	0.07
Wound infection	3	8	0.50
Pneumonia	1	3	0.87
Urinary tract infection	1	1	1.00
Deep vein thrombosis	1	0	0.86
Heart failure	0	2	0.63
Myocardial infarction	1	0	0.86
Hospital stay	31.2 ± 11.3	36.0 ± 14.6	0.07

AIT: Air insufflation test.

Intraoperative outcomes

The intraoperative data and pathological diagnoses are listed in Table 2. The two groups were similar in terms of operative factors, pancreatic factors and pathological diagnoses. Most of the operations (96/111, 86.5%) were performed for malignant diseases.

The AIT was successfully performed in all 46 of the patients in the AIT group. Pancreatic leakage was found in 10 patients, and immediate repair or re-anastomosis was performed.

Postoperative outcomes

The overall mortality, morbidity, and PF rates of all of the patients were 1.8%, 48.6%, and 26.1%, respectively. No significant differences were found in the mortality rate (AIT group *vs* non-AIT group, 2.2% *vs* 1.5%, P = 1.00) or the overall complication rate (AIT group *vs* non-AIT group, 43.5% *vs* 52.3%, P = 0.36) between the two groups (Table 3).

PF was the most frequent complication after PD. The PF rate (AIT group *vs* non-AIT group, 19.6% *vs* 30.8%, P = 0.19) and the prevalence of grade A PF (AIT group *vs* non-AIT group, 13.0% *vs* 7.7%, P = 0.54) were comparable between the two groups. However, the incidence of clinical PF was significantly lower in the AIT group, compared with the non-AIT group (AIT group *vs* non-AIT group, 6.5% *vs* 23.1%, P = 0.02). In addition, 2 patients (of the 10 who experienced repair or re-anastomosis) suffered from grade A PF. Moreover, the overall PF rate, grade A PF rate and clinical PF rate of the remaining 36 patients in the AIT group who did not receive repair or re-anastomosis were 19.4% (n = 7), 11.1% (n = 4) and 8.3% (n = 3), respectively. Interestingly, the statistical analysis revealed that the overall PF rate, grade A PF rate and clinical PF rate of the 36 patients

Table 4 Univariate analysis of risk factors for clinical pancreatic fistula

	Clinical PF group (n = 18)	Non-clinical PF group (n = 93)	P value
Age (yr)	56.6 ± 9.8	56.4 ± 10.3	0.96
Gender			0.58
Male	10	45	
Female	8	48	
ASA			0.55
Ⅱ	17	80	
Ⅲ	1	13	
ALT (U/L)	263.8 ± 298.9	191.8 ± 179.7	0.17
AST (U/L)	171.6 ± 232.3	135.8 ± 130.2	0.36
Preoperative bilirubin (μmol/L)	185.8 ± 159.8	151.2 ± 149.1	0.38
Preoperative albumin (g/L)	68.1 ± 6.8	65.6 ± 8.2	0.23
Presence of comorbid illness	5	38	0.30
Mellitus diabetes	0	11	0.27
Chronic pulmonary disease	1	5	1.00
Artery hypertension	1	16	0.37
Coronaropathy	3	6	0.33
Preoperative biliary drainage			0.25
Yes	11	43	
No	7	50	
Smoking			0.84
Yes	5	22	
No	13	71	
Operative factors			
Combined vascular resection	1	2	0.42
Duration of operation (min)	392.8 ± 103.3	349.2 ± 93.2	0.08
Estimated blood loss (mL)	428.9 ± 272.6	290.2 ± 144.0	0.05
Blood transfusion cases	7	26	0.35
Pancreatic factors			
Pancreatic duct diameter			0.02
⩽ 3 mm	16	57	
> 3 mm	2	36	
Pancreatic texture			0.66
Soft	15	70	
Hard	3	23	
Pancreatic anastomosis			0.02
Duct to mucosa anastomosis	2	38	
Invagination anastomosis	16	55	
Air insufflation test			0.02
Yes	3	43	
No	15	50	
Pathological diagnoses			0.96
Malignant disease	15	81	
Pancreatic carcinoma	4	20	1.00
Ampullar carcinoma	1	17	0.32
Cholangiocarcinoma	4	15	0.78
Duodenal carcinoma	6	17	0.26
Benign diseases	3	12	

ASA: American Society of Anesthesiologists; ALT: Alanine aminotransferase; AST: Aspartate transaminase; PF: Pancreatic fistula.

were similar to those of the 65 patients in the non-AIT group (P > 0.05), supporting the contribution of the repair or re-anastomosis after the air insufflation test to the significant reduction of clinical PF in the AIT group. No special treatments were performed for the patients with grade A PF. Radiologic or surgical intervention for PF was required for 1 patient in the AIT group and 8 patients in the non-AIT group. Other patients were treated conservatively with enteral or parenteral nutrition, a somatostatin analog and antibiotics. The length of hospital stay was 34.0 ± 14.5 d for all of the patients, and the length of hospital stay for the AIT group was shorter than for the non-AIT group. However, this difference was only a statistical trend (AIT group *vs* non-AIT group, 31.2 ± 11.3 d *vs* 36.0 ± 14.6 d, P = 0.07).

Two patients died during this clinical study (AIT group *vs* non-AIT group, 1 *vs* 1, P = 1.00), and they both died due to intra-abdominal infection and hemorrhage related to PF.

Risk factors of postoperative PF

Multiple variables related to clinical PF were statistically analyzed with univariate analysis (Table 4), and four risk factors were identified: estimated blood loss; pancreatic duct diameter ⩽ 3 mm; invagination anastomosis technique; and not undergoing the air insufflation test.

These variables were further analyzed in multivariate analysis. The estimated blood loss, pancreatic duct diameter ⩽ 3 mm and not undergoing the air insufflation test were identified as independent risk factors (P = 0.02, 0.00 and 0.00; OR = 1.00, 28.73 and 18.00; and 95%CI: 1.00-1.01, 4.39-188.17 and 3.49-92.96, respectively) for clinical PF.

DISCUSSION

Despite the evolution of surgical techniques, the PF rate after PD has remained high. PF is one of the most frequent lethal complications after PD. Palani Velu *et al*[27] reported that serum amylase on the night of surgery predicted clinically significant PF after PD. Molinari *et al*[28] and Hashimoto *et al*[29] both demonstrated that the amylase levels of PF patients were significantly higher than those of non-PF patients on the first postoperative day. These reports indicated that some PFs might be caused by unsuccessful anastomoses that went undiscovered during surgery, leading to the elevation of amylase levels in the drainage fluid on the first night and first postoperative day. In the present study, we used the air insufflation test to investigate the pancreatojejunal anastomosis expecting to discover the leakage during operation and repair it immediately. The air insufflation test could detect an incomplete anastomosis and was more sensitive than visual examination, with which it was often difficult to find minor leakage because of hemorrhage.

The air insufflation test did not prolong the operation time; rather, it improved the patient outcomes significantly and was simple and effective. However, there were some particularly important points to note during the testing process. We suggest that the air should be gently injected, and the pressure within the jejunum stump should be monitored throughout the entire process. Acute pancreatitis can be caused if too much air is injected, or the air is injected too quickly.

When re-anastomosis is needed, a duct to mucosa anastomosis should usually be changed to an invagination anastomosis if the leakage cannot be resolved after twice re-anastomoses. In the present study, anastomotic revision was conducted in 10 patients. Of these patients, one patient experienced three times pancreaticojejunal re-anastomoses, and PF was not observed until the patient was discharged.

In accordance with the ISGPF definition[24], the PFs in this study were classified as grade A, B or C, based on the clinical impact on the patients' in-hospital outcomes. Grade A is also called "transient fistula," and it has no clinical impact[24]. Poor patient outcomes have primarily been caused by grade B and grade C PFs. Fuks *et al*[12] examined grade C PFs in a multiple center study. They reported a reoperation rate of 97% and a mortality rate of 38.8% for grade C patients. In this study, two patients died postoperatively, and both deaths were caused by grade C PFs. The clinical PF rate (grade B and grade C) was significantly reduced in the AIT group compared with the non-AIT group (AIT group *vs* non-AIT group, 6.5% *vs* 23.1%, $P = 0.02$), and the radiologic or surgical intervention rate was also reduced. However, no significant difference was found (AIT group *vs* non-AIT group, 2.2% *vs* 12.3%, $P = 0.12$).

The current study also identified pancreatic duct diameter less than 3 mm and estimated blood loss as independent risk factors for clinical PF, consistent with previous studies[5,6,8,30]. Duct to mucosa anastomosis was identified as a risk factor for clinical PF in univariate analysis. Different anastomosis techniques have been reported in previous studies. Pancreatic duct to jejunal mucosa anastomosis has been advocated in many series[31,32]. Tsuji *et al*[33] reported on 300 patients who underwent PD, and the incidence of fistula in the patients who received continuous suture of the pancreatic duct to the jejunal mucosa (4.2%) was significantly less than that of the patients who received interrupted sutures (17.2%) ($P < 0.01$). Lee *et al*[34] revealed that continuous sutures for the outer layer of the pancreaticojejunostomy could significantly reduce the PF rate, compared with interrupted sutures. Interrupted suturing of the duct to the mucosa and to the outer layer of the pancreaticojejunostomy was performed in our study, and the clinical PF rate was also statistically less than with the invagination technique. The pancreatic texture has been reported as a risk factor for PF in many studies[2,8]. The incidence of soft remnant pancreas in the clinical PF group was higher than in the non-clinical PF group (83.3% *vs* 75.3%), but the difference was not statistically significant ($P > 0.05$).

In conclusion, the present study confirmed the efficacy of the air insufflation test in preventing clinical PF. It was simple to conduct and could significantly reduce the incidence of clinical PF. Estimated blood loss, pancreatic duct diameter $\leqslant$ 3 mm and not undergoing the air insufflation test were identified as independent risk factors for clinical PF in multivariate analysis. This study was retrospective and carried multiple biases. Due to the relatively small number of patients included, additional research is needed to confirm the efficacy of the air insufflation test.

COMMENTS

Background

Pancreaticoduodenectomy (PD) has been the standard operation for the resection of periampullary diseases. Unfortunately, regardless of improved surgical techniques and postoperative care, the pancreatic fistula (PF) rate after PD has remained approximately 30%. Moreover, PF has been a primary factor linked with death after PD in some case studies.

Research frontiers

Different risk factors have been identified to predict PF, and numerous strategies have been reported to prevent PF. However, their efficacy has been controversial, and these strategies have not been routinely used in most medical centers. More effective and confirmative strategies are needed to reduce the incidence of PF.

Innovations and breakthroughs

Although different strategies have been adopted to prevent PF, none of them can identify whether pancreatic leakage occurs during surgery. The air insufflation test detected pancreatic leakage during the surgery, and its efficacy was also confirmed. Additionally, the risk factors related to PF were also identified in this article.

Applications

The air insufflation test could detect pancreatic leakage during PD. In the future, it might be used to identify whether leakage exists in other anastomoses, such as the hepaticojejunal anastomosis.

Terminology

PF is defined as drainage output of any measurable volume of fluid on or after postoperative day 3 with an amylase content greater than 3 times the serum amylase activity.

Peer-review

Interesting study looking at the use of insufflation of the pancreaticojejeunostomy with air to test for leaks to attempt to minimize the risk of PF. This is a useful contribution to the surgical literature.

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ORIGINAL ARTICLE

Clinical Trials Study

Effects of oral tacrolimus as a rapid induction therapy in ulcerative colitis

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Abstract

AIM: To determine the efficacy and safety of rapid induction therapy with oral tacrolimus without a meal in steroid-refractory ulcerative colitis (UC) patients.

METHODS: This was a prospective, multicenter, observational study. Between May 2010 and August 2012, 49 steroid-refractory UC patients (55 flare-ups) were consecutively enrolled. All patients were treated with oral tacrolimus without a meal at an initial dose of 0.1 mg/kg per day. The dose was adjusted to maintain trough whole-blood levels of 10-15 ng/mL for the first 2 wk. Induction of remission at 2 and 4 wk after tacrolimus treatment initiation was evaluated using Lichtiger's clinical activity index (CAI).

RESULTS: The mean CAI was 12.6 ± 3.6 at onset. Within the first 7 d, 93.5% of patients maintained high trough levels (10-15 ng/mL). The CAI significantly decreased beginning 2 d after treatment initiation. At 2 wk, 73.1% of patients experienced clinical responses. After tacrolimus initiation, 31.4% and 75.6% of patients achieved clinical remission at 2 and 4 wk, respectively. Treatment was well tolerated.

CONCLUSION: Rapid induction therapy with oral tacrolimus shortened the time to achievement of appropriate trough levels and demonstrated a high remission rate 28 d after treatment initiation. Rapid induction therapy with oral tacrolimus appears to be a useful therapy for the treatment of refractory UC.

Key words: Ulcerative colitis; Tacrolimus; Rapid induction therapy; Steroid-refractory ulcerative colitis; Inflammatory bowel disease

Core tip: A prospective, multicenter, observation study was conducted to determine the efficacy and safety of rapid induction therapy with oral tacrolimus without meal in patients with steroid-refractory ulcerative colitis. Rapid induction therapy could achieve the appropriate trough level (10-15 ng/mL) within the first 7 d and revealed a high remission rate 28 d after the initiation of the treatment. Treatment was well tolerated. Rapid induction therapy with oral tacrolimus appears to be a useful therapy for the treatment of refractory ulcerative colitis.

Kawakami K, Inoue T, Murano M, Narabayashi K, Nouda S, Ishida K, Abe Y, Nogami K, Hida N, Yamagami H, Watanabe K, Umegaki E, Nakamura S, Arakawa T, Higuchi K. Effects of oral tacrolimus as a rapid induction therapy in ulcerative colitis. *World J Gastroenterol* 2015; 21(6): 1880-1886 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i6/1880.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i6.1880

INTRODUCTION

Ulcerative colitis (UC) is an idiopathic inflammatory bowel disease (IBD) characterized by a chronic relapsing/intermittent clinical course. Aminosalicylates are typically used as first-line treatment for patients with UC, while steroids are usually considered to be second-line treatment and are used to induce remission when remission cannot be achieved with aminosalicylates[1]. Because steroids have a rapid onset of action and are highly effective, they are reserved for disease that fails to respond to primary therapy in patients with severe UC. However, these agents are associated with considerable systemic adverse effects[2]. Nevertheless, approximately 20% of patients with UC have chronically active disease that requires several courses of steroids[3]. As a result, many steroid-dependent/-resistant patients experience severe complications of steroid treatment before stable remission can be achieved[3,4].

Calcineurin inhibitors, such as cyclosporine A (CsA) and tacrolimus, inhibit the production of interleukin-2 (IL-2) and activation of T lymphocytes[5]. Since these agents have a rapid onset of action and are highly effective in patients with refractory UC, they are approved as an alternative treatment option for refractory UC under the national health insurance system in Japan[6]. Previous reports have demonstrated the dose-dependent efficacy and safety of oral tacrolimus for remission-induction therapy in refractory UC[7,8]. With respect to efficacy, the optimal target tacrolimus blood concentration appears to be 10-15 ng/mL[7,8]. However, since oral tacrolimus has a slower onset of action compared to intravenous CsA[6], we have occasionally had severe UC patients who did not demonstrate improvement prior to achieving the appropriate trough level with oral tacrolimus using standard dosing (initial dose of 0.025 mg/kg twice daily). Even when the starting dose of tacrolimus is set to 0.1 mg/kg per day, it still takes more than 7 d to achieve the target tacrolimus blood concentration, because food intake is known to reduce serum levels of tacrolimus due to its low absorption rate[6,7]. Therefore, rapid induction therapy with oral tacrolimus, starting at 0.1 mg/kg per day without meals, should be recommended as a salvage therapy for patients with steroid-dependent/resistant UC. Meanwhile, higher tacrolimus starting doses have been reported to be associated with nephrotoxicity and to have no apparent therapeutic advantage over lower doses, suggesting that lower doses should be used to avoid adverse events[8]. However, thus far, no prospective studies designed to evaluate the efficacy and safety of rapid induction therapy with oral tacrolimus have been conducted. Therefore, we have conducted such a prospective, multicenter study, and report herein the efficacy and safety of rapid induction therapy with oral tacrolimus in steroid-refractory UC patients.

MATERIALS AND METHODS

Study design and patients

This was a prospective, multicenter, observational study involving three Japanese academic centers: Hyogo College of Medicine, Osaka City Graduate School of Medicine, and Osaka Medical College. Patients admitted to hospitals for the treatment of steroid-resistant or steroid-dependent moderate/severe UC were eligible. The inclusion criteria were as follows: (1) > 16 years of age at admission and capable of providing written informed consent; (2) diagnosis established according to standardized criteria with prior clinical assessments, radiology, endoscopy, and histology[9]; (3) steroid-resistant or steroid-dependent UC; and (4) active colitis as assessed by a Mayo score of 8-12[10]. The exclusion criteria were as follows: (1) under 16 years of age or unable to give informed consent; (2) prior abdominal surgery; (3) pregnant, at risk of pregnancy, or breast feeding; and (4) presence of active extra-intestinal infection, liver or kidney failure, or suspicious for diabetes. All patients were hospitalized and had left-sided UC (except for ulcerative proctitis) or extensive UC. The extent of colonic involvement was determined by total colonoscopy.

Patients were classified as having steroid-resistant or steroid-dependent disease in accordance with the definitions previously published by Ogata *et al*[7] Steroid resistance was defined as a lack of response to oral or intravenous steroid therapy (daily prednisolone dose > 30 mg) over at least 2 wk, and steroid dependence was defined as recurrent flare-up on steroid reduction or withdrawal, or chronic active UC for > 6 mo or frequent recurrence (more

Table 1 Dose adjustment of rapid induction of oral tacrolimus

Trough concentration	< Day 4	Trough concentration	Days 4-14
> 40 ng/mL	0 mg/d	> 40 ng/mL	0 mg/d
25-40 ng/mL	-33%	30-40 ng/mL	-50%
		20-30 ng/mL	-25%
10-25 ng/mL	0%	10-20 ng/mL	0%
< 10 ng/mL	33%	< 10 ng/mL	25%

than once a year, or three times or more every two years, regardless of intensive medical therapy)[7,11]. Patients were permitted to continue taking drugs containing 5-aminosalicylic acid during this study. Patients who were already taking steroids and/or immunosuppressant agents (azathioprine or 6-mercaptopurine) were also permitted to continue receiving these medications. However, the dosages of these agents were not allowed to be increased once the patients entered into this study. In addition, cytapheresis was prohibited during the study period.

This study was reviewed and approved by the ethical committee of each academic center. All patients were informed of the potential risks and benefits of tacrolimus therapy and provided signed informed consent forms before undergoing any procedures.

Administration and dose adjustment

All patients were hospitalized at the time of initiation of tacrolimus therapy and given oral tacrolimus without a meal at an initial dose of 0.1 mg/kg per day, given in twice-daily divided doses. Blood was collected to determine the tacrolimus whole-blood trough concentrations at 24 h and at 2, 3, 4, and 7 d and every 7 d thereafter after the initial dose. The dose was adapted to maintain trough whole-blood levels of 10-15 ng/mL for the first 2 wk. The doses were adjusted using the equations shown in Table 1. After a high trough concentration (10-15 ng/mL) was achieved, patients could resume receiving meals. Beginning at 2 wk after the initiation of tacrolimus therapy, tacrolimus whole-blood trough concentrations were gradually maintained at a lower level of 5-10 ng/mL.

Symptom assessment and study endpoints

The primary endpoint of this study was the proportion of patients with improvement, [defined as a clinical response or clinical remission, based on Lichtiger's clinical activity index (Lichtiger score)]. The secondary endpoint was safety. Patients were hospitalized until their clinical conditions had stabilized and their tacrolimus levels were within the therapeutic range. Lichtiger scores were obtained at 1, 2, 3, 5, 7, 10, 14, 21, and 28 d after the initiation of tacrolimus treatment. Response was defined as a Lichtiger score < 10 and a decrease $\geqslant$ 3 (in the case of patients with a Lichtiger score less than 10 on admission, response was defined as a Lichtiger score decrease > 3), and clinical remission was defined as a Lichtiger score $\leqslant$ 4[12,13]. Response rate and remission rate were defined as the proportion (%) of patients achieving response or remission, respectively[14]. Trough whole-blood levels and biochemical values, including serum creatinine and fasting blood glucose levels, were also measured.

Table 2 Clinical details of patients

Patients, *n*	49
Attacks, *n*	55
Sex, M/F	25/24
Age (mean ± SD)	43.8 ± 16.0
Extent of disease (left sided UC/extensive UC), *n*	5/44
CAI (mean ± SD)	12.6 ± 3.9
Steroid resistant/dependent, *n*	38 cases, 42 flare-ups /13 cases, 13 flare-ups

UC: Ulcerative colitis; CAI: Clinical activity index.

Statistical analysis

Continuous data ware statistically analyzed using Student's *t*-test. The Wilcoxon test was used to analyze clinical scores (*i.e.*, Lichtiger scores). Results are expressed as the mean ± SD. *P*-values < 0.05 were considered to be statistically significant. All calculations were made using the Statview system (SAS Institute, Cary, NC, United States).

RESULTS

Patient characteristics

This study was performed between May 2010 and August 2012. A total of 49 patients (55 flare-ups) with steroid-resistant or steroid-dependent UC in three Japanese academic centers were enrolled. Baseline patient characteristics are shown in Table 2. According to the Montreal classification[15], 44 patients (89.8%) were afflicted with extensive UC, and 5 patients (10.2%) were afflicted with left-sided UC. The mean clinical activity index (CAI) was 12.6 ± 3.6 at onset, and all enrolled patients had a Lichtiger score $\geqslant$ 6. Of 49 patients, 4 patients were enrolled into this study twice, and 1 patient was enrolled three times, due to relapse. Two patients had steroid-resistant disease at first admission and steroid-dependent disease at second admission. Therefore, of the 49 patients, 38 patients (42 attacks) had steroid resistance and 13 patients (13 attacks) had steroid dependence; 2/49 patients were included in both groups because they had two different types of flare-ups.

Administration and dose adjustment

Daily oral tacrolimus dosing was started at 6.47 ± 1.18 mg (0.12 ± 0.03 mg/kg) and significantly increased 1 d after initiation of treatment. From day

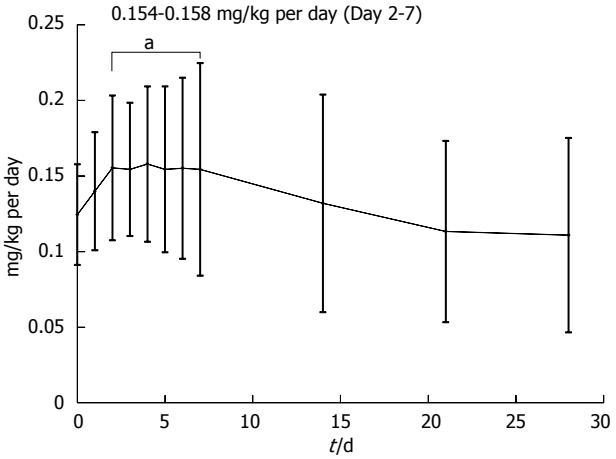

Figure 1 Daily dosage of oral tacrolimus. $^{a}P < 0.05$ *vs* day 0.

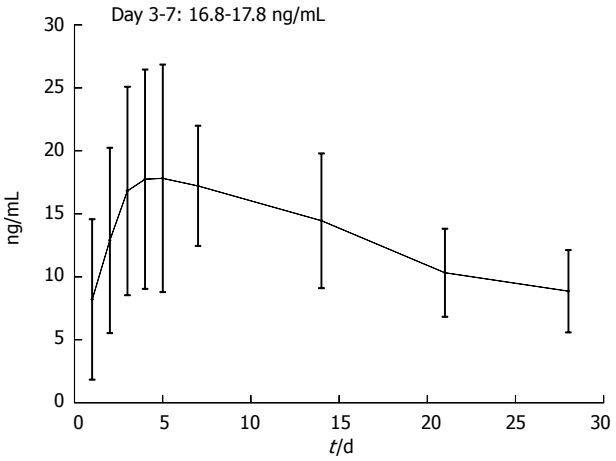

Figure 2 Mean trough whole-blood levels.

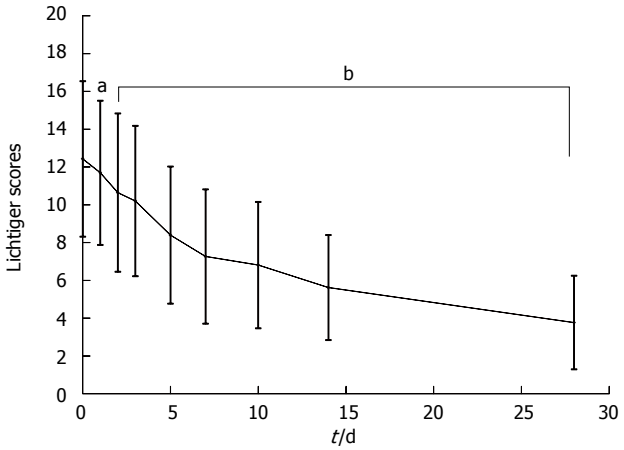

Figure 3 Evolution of Lichtiger scores following treatment with oral tacrolimus. $^{a}P < 0.05$ *vs* day 0, $^{b}P < 0.01$ *vs* day 0.

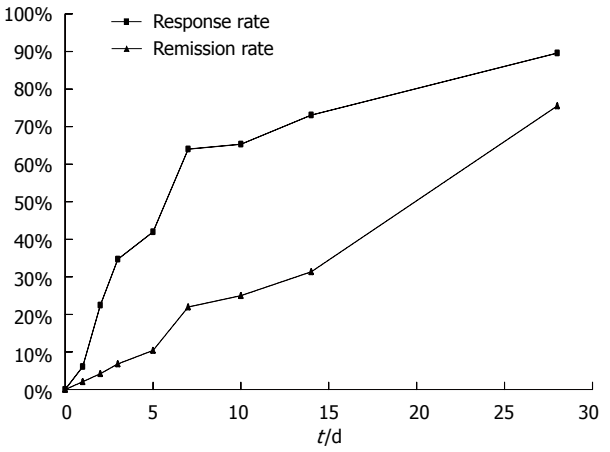

Figure 4 Clinical responses following treatment with oral tacrolimus.

2 to day 7, relatively stable tacrolimus doses were required to maintain trough whole-blood levels of 10-15 ng/mL (8.09 ± 3.47 to 8.31 ± 2.64 mg/d and 0.15 ± 0.04 to 0.16 ± 0.05 mg/kg per day) (Figure 1). Mean trough whole-blood levels reached a maximum on day 2 (12.89 ± 7.35 ng/mL) and 93.5% of patients were able to maintain high trough levels (> 10 ng/mL) within the first 7 d of treatment (Figure 2). Mean trough whole-blood levels gradually decreased to 8.85 ± 3.27 ng/mL at day 28.

Clinical response

The mean Lichtiger score at the time of treatment initiation was 12.6 ± 3.6. The Lichtiger score decreased significantly beginning 2 d after the initiation of tacrolimus treatment (Figure 3). Two weeks after initiation of therapy, rapid induction therapy with oral tacrolimus resulted in a clinical response in 73.1% of patients and a clinical remission in 31.4% of patients. Four weeks after initiation of therapy, clinical response and remission were observed in 89.6% and 75.6% of patients, respectively (Figure 4). Within 28 d of tacrolimus treatment, colectomy was required in 3 patients due to their disease becoming refractory to tacrolimus. No significant differences in Lichtiger score, trough levels, clinical response, or clinical remission were observed between patients with steroid-resistant and steroid-dependent UC.

Adverse effects

The mean serum creatinine level did not significantly change during tacrolimus treatment. Although 48.6% (18/37) of the patients had at least one elevated glucose (> 120 mg/dL) measured while on tacrolimus treatment, mean fasting blood glucose level was significantly lower at day 21 compared with that on day 0 (86.0 ± 21.4 mg/dL and 107.3 ± 22.9 mg/dL, respectively; $P = 0.012$) (Figure 5). Other documented clinical reactions and laboratory abnormalities thought to be related to tacrolimus included tremors (35.7%, 15/42), headache (9.5%, 4/42), nausea (7.1%, 3/42), and hypomagnesemia (74.1%, 20/27, 1.56 ± 0.26 mg/dL) (Table 3). Overall, treatment was well tolerated, with no patient requiring treatment disruption or termination due to

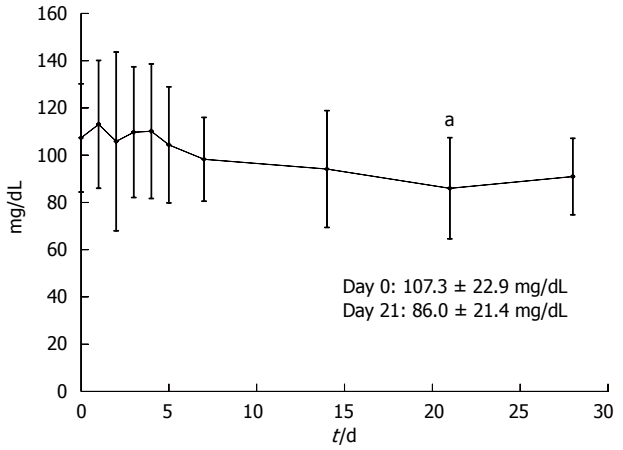

Figure 5 Blood glucose level following treatment with oral tacrolimus. [a]*P* < 0.05 *vs* day 0.

adverse effects.

DISCUSSION

To our knowledge, this is the first prospective multicenter study that has evaluated the effect of rapid induction therapy with oral tacrolimus in patients with refractory UC. The present results have shown that rapid induction therapy with oral tacrolimus was well tolerated and yielded a high clinical response rate within 2 wk and a high clinical remission rate within 4 wk after initiation of treatment. These results suggest that rapid induction therapy with oral tacrolimus should be an option for patients with refractory UC.

Tacrolimus is a macrolide immunosuppressant that is structurally similar to rapamycin and has been found to have potent immunosuppressive properties that are 10- to 100-fold more potent in inhibiting lymphocyte activation than CsA[16-18]. Since less variability in absorption and serum levels is observed among patients treated with tacrolimus compared to those who receive oral CsA, tacrolimus has been suggested to be more easily and safely administered to patients with refractory UC than CsA. Ogata *et al*[7] conducted the first randomized controlled trial that demonstrated the efficacy of oral tacrolimus in refractory UC patients. A total of 68.4% of patients in the high trough concentration (10-15 ng/mL) group improved within 2 wk after administration of tacrolimus, whereas only 38.1% of patients in the low trough concentration group experienced disease improvement. Thus far, several uncontrolled[15-17,19-21] and two placebo-controlled studies[7,8] have demonstrated that tacrolimus can induce remission in both adults and children, and these reports suggested that tacrolimus had a trough concentration-dependent effect, with the optimal target range appearing to be 10-15 ng/mL with a relatively short period of efficacy. Regarding long-term efficacy in patients with refractory UC, Yamamoto *et al*[13] investigated the efficacy of tacrolimus as maintenance therapy for patients with refractory UC and reported that the cumulative colectomy-free survival rate was 62% at 65 mo. The colectomy-free survival rate was significantly higher in patients who responded to tacrolimus within 30 d than in those who did not. We previously examined the short-term efficacy of tacrolimus in refractory UC and found that the clinical response rate at 4 wk after initiation of tacrolimus treatment correlated with the mean trough level at 8-21 d after treatment initiation[22]. The primitive trough raised within 5 d after administration is considered to be important for obtaining the appropriate trough level at 8 d after tacrolimus administration. Since oral tacrolimus has a slower onset of action compared to intravenous CsA, and food intake is known to reduce tacrolimus serum trough levels due to its low absorption rate, oral tacrolimus takes more than 7 d to reach high trough levels. Therefore, we have occasionally had severe UC patients who did not demonstrate improvement prior to achieving the appropriate trough level with oral tacrolimus using standard dosing. Indeed, Schmidt *et al*[23] also evaluated the short-term efficacy of oral tacrolimus in moderate to severe steroid-refractory UC using a starting dose of 0.1 mg/kg per day, and reported that clinical remission was achieved in 52.3% of patients, and 14% of patients required colectomy. In the present study, a high clinical response rate was observed at 2 wk after initiation of treatment (73.1%, 38/52) and a high clinical remission rate was achieved at 4 wk (75.6%, 34/45). Although we cannot directly compare the results of Schmidt *et al*[23] with those of the present study due to differences in patient baseline characteristics, rapid induction therapy with oral tacrolimus in the present study appears to yield high remission rates in patients with steroid-refractory UC. Strict dose adjustment of tacrolimus in the early treatment phase has been suggested to provide excellent clinical outcomes and to avoid the need for surgery for refractory UC, because the efficacy of tacrolimus depends on trough blood levels[24]; therefore, we suggest that whole-blood tacrolimus levels should be measured in all patients, and early dose increases should be made as part of rapid induction therapy. In the present study, 0.15-0.16 mg/kg per day of oral tacrolimus was needed to achieve appropriate trough levels; the mean trough

Table 3 Adverse responses *n* (%)

Adverse responses	Value
Hypomagnesemia (< 1.7 mg/dL)	20 (74.1)
Elevated blood glucose (> 120 mg/dL)	18 (48.6)
Tremor	15 (35.7)
Headache	4 (9.5)
Nausea	3 (7.1)
Elevated serum creatinine (> 1.2 mg/dL)	2 (5.4)

level reached high on day 2; and 93.5% of patients could maintain high trough levels within the first 7 d of treatment. Thus far, no studies have evaluated the efficacy of, or dose adjustment for rapid induction therapy with high-dose oral tacrolimus without meals.

Similar to CsA, tacrolimus is known to be associated with many adverse effects, such as infections, renal dysfunction, hypertension, and neurological toxicity. However, these effects are generally mild and reversible. In this study, mean serum creatinine was not significantly elevated during 4 wk of treatment. Interestingly, the mean fasting blood glucose level was significantly lower 21 d after the initiation of treatment as the CAI improved. With regard to other adverse effects, many patients developed hypomagnesemia (74.1%, 1.56 ± 0.26 mg/dL), at a frequency similar to that reported in previous studies (33.3%-87.5%)[7,16]. Although 48.6% (18/37) of the patients had at least one elevated glucose measured during this study, the mean fasting blood glucose level was significantly lower at day 21 compared with that on day 0. Benson also reported that 62.5% of patients had elevated glucose and most of them were on corticosteroid therapy at that time[16]. We therefore consider that it was likely that the hyperglycemia observed was not related to tacrolimus treatment. Tremor appeared to be increased (35.7%) compared with that reported previously (9.4%-19.0%)[7,8,16,25]. However, no significant clinical symptoms were observed during treatment, and no patient discontinued oral tacrolimus therapy due to adverse effects. Thus, we consider that rapid induction therapy with oral tacrolimus will play a major role in safely inducing remission in patients with refractory UC.

Regarding starting dose of oral tacrolimus and dose adjustment, 29.1%-36.4% of patients needed to do dose adjustment from day 1 to day 4 (data was not shown) and finally daily treatment of oral tacrolimus at dose of 0.15-0.16 mg/kg was needed to achieve appropriate trough levels. Therefore, we consider that oral tacrolimus at an initial dose more than 0.1 mg/kg per day may decrease the number of times of dose adjustment and be more suitable for the patients with refractory UC.

The uncontrolled study design was a limitation of this study. To clarify the efficacy of rapid induction therapy with oral tacrolimus, further study in which patients are randomized to either rapid induction or standard induction is necessary. Nonetheless, this is the first study to confirm that rapid induction therapy with oral tacrolimus is a safe and highly effective treatment for patients with refractory UC. Although any long-term effects of this treatment method remain unclear, the rapid induction therapy administered in this study may be useful for the treatment of patients with refractory UC. Further studies are needed to evaluate the long-term outcomes of rapid induction therapy with oral tacrolimus in patients with UC.

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COMMENTS

Background

Tacrolimus has a rapid onset of action and is highly effective in refractory ulcerative colitis (UC) patients. However, the authors have occasionally have had severe UC patients who did not demonstrate improvement prior to achieving the appropriate trough level with oral tacrolimus using standard dosing (0.025 mg/kg twice daily).

Research frontiers

Because food intake is known to reduce serum levels of tacrolimus due to its low absorption rate, rapid induction therapy with oral tacrolimus, starting at 0.1 mg/kg per day without meals should be recommended as a salvage therapy for patients with refractory UC. However, the efficacy and safety of rapid induction therapy with oral tacrolimus has been unknown.

Innovations and breakthroughs

This is the first prospective multicenter study that has evaluated the effect of rapid induction therapy with oral tacrolimus in patients with refractory UC. The present results have shown that rapid induction therapy with oral tacrolimus was well tolerated and yielded a high clinical response/remission rate.

Applications

Although any long-term effects of this treatment method remain unclear, the rapid induction therapy administered in this study may be useful for the treatment of patients with refractory UC. The results of this study may help physicians decide how to use oral tacrolimus for the patients with refractory UC.

Peer-review

This is an interesting prospective, multicenter study assessing the efficacy and safety of rapid induction therapy with oral tacrolimus in refractory UC patients.

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ORIGINAL ARTICLE

Observational Study

Assessing cultural competency skills in gastroenterology fellowship training

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Abstract

AIM: To assess and teach cultural competency skills at the fellowship training level through the use of objective structured clinical examinations (OSCEs).

METHODS: We revised four scenarios to infuse a specific focus on cross-cultural care, and to render them appropriate for gastroenterology fellows. Three are discussed here: (1) Poor Health Literacy; (2) Disclosing/Apologizing for a Complication to a Patient Who Mistrusts the Healthcare System; and (3) Breaking Bad News to a Fatalistic Patient. A fourth case emphasizing shared decision-making will be described elsewhere. Four stations were completed by fellows and observed live by four faculty members, and the fellows' performance was assessed.

RESULTS: Eleven fellows from four programs participated in the four OSCE. In the "Poor Health Literacy" case, 18% (2/11) of participants recognized that the standardized patient (SP) had below-basic health literacy. None successfully evaluated the SP's reading skills in a culturally-sensitive manner. In "Disclosing/Apologizing for a Complication", 4/11 (36%) personally apologized for the complication. 1/11 recognized the SP's mistrust of the medical system. With "Breaking Bad News", 27% (3/11) explored the patient's values to identify her fatalistic beliefs.

CONCLUSION: OSCEs can be used to assess deficiencies in culturally-competent care at the fellowship level. OSCEs also afford fellowships the opportunity to inform future training curricula.

Key words: Patient care; Physician-patient relations; Gastroenterology; Cultural competency; Education; Patient care; Health literacy; Health care; Graduate; Objective structured clinical examination; Trainees

Core tip: Cultural competency is an integral skill set vital to a fruitful physician-patient relationship. However, we lack tools necessary to assess and teach such skills, especially at the fellowship level. We designed an objective structured clinical examination (OSCE) on specific criteria essential to the delivery of culturally-competent care. Our findings suggest that although the participating trainees' can adequately provide some aspects of culturally-competent care, their ability to wholly execute such care is subpar. The current fellowship program curricula may not adequately prepare its trainees to successfully employ culturally-competent care, and OSCEs provide a means to assess and teach this complex skill set.

Balzora S, Abiri B, Wang XJ, McKeever J, Poles M, Zabar S, Gillespie C, Weinshel E. Assessing cultural competency skills in gastroenterology fellowship training. *World J Gastroenterol* 2015; 21(6): 1887-1892 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i6/1887.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i6.1887

INTRODUCTION

There is an increasing recognition of the need for medical training programs to improve physician-patient communication and to counter the ethnic and racial disparities that exist in health care, which potentially result in poorer patient satisfaction and outcomes[1]. In 2001, the Accreditation Council for Graduate Medical Education (ACGME) identified interpersonal and communication skills, and professionalism as two of its six core competencies for residents and fellows. Cultural competency is defined as the ability of health care professionals to communicate with and effectively provide high-quality care to patients from diverse socio-cultural backgrounds[2]. Objective structured clinical examinations, or OSCEs, serve as a valid tool to measure the performance of trainee physicians in various aspects of patient care[3-5]. This observation has been extended to gastroenterology training with great success[6-8]. Still, to date, studies to assess the competency level of gastroenterology (GI) fellows in providing cross-cultural care have yet to be performed.

Therefore, the aim of this project was to use OSCEs to formally assess the ability of gastroenterology fellows who train in an urban, socioeconomically diverse population such as New York City, to implement cultural competency as a skill in a variety of challenging clinical scenarios. Here, we present three clinical cases that we have modified from cases previously described that address specific facets of cultural competency: Poor Health Literacy, Disclosing and Apologizing for a Complication to a Patient Who Mistrusts the Healthcare System, and Breaking Bad News to a Religiously Fatalistic Patient.

MATERIALS AND METHODS

Participants

Using validated OSCE checklists, we revised the clinical elements of 3 previously described cases (Health literacy, Disclosing and apologizing for a complication, and Breaking bad news) to make them more suitable for GI fellows, and to add a specific focus on various aspects of cultural competency. Four faculty members from two GI training programs in New York City (NYU School of Medicine and Icahn School of Medicine at Mount Sinai) observed the four-station OSCE and 11 fellows (from the programs listed above, as well as Lenox Hill Hospital and St. Luke's-Roosevelt Hospital Center) participated. The standardized patients (SPs) were trained for 3 h with scripts and role-playing to standardize their case portrayals and fellow ratings.

OSCE cases

Poor Health Literacy (Health Literacy Case): The clinical scenario was of a 27 year-old new mother of Chinese descent with below-basic health literacy who was diagnosed with chronic hepatitis B virus infection during her pregnancy. The goals of the GI fellow were the following: identify the patient as health-illiterate, and successfully convey health information to her about her disease.

Disclosing and Apologizing for a Complication to a Patient Who Mistrusts the Healthcare System (Apologizing for a Complication Case): The clinical scenario was of a 50-year-old African-American male patient with a harbored mistrust of the healthcare system who undergoes an average-risk screening colonoscopy with polypectomy, subsequently complicated by a perforation. The goals of the GI fellow were the following: recognize this patient's mistrust of the medical system and how it impacted his response to the complication, admit that a medical error was made, and regain the patient's trust, and have him willingly agree to stay for follow-up care.

Breaking Bad News to a Religiously Fatalistic Patient (Breaking Bad News Case): The clinical scenario was of a 54 year-old African-American female patient with a family history of a first-degree relative with colon cancer who had just undergone a screening colonoscopy during which a likely colon cancer is found. When informed of this finding, she believes that succumbing to colon cancer is God's will for her and she refuses to consider further work-up or

Table 1 Health literacy *n* (%)

Competency areas and specific skills	Distribution of responses, *n* = 11		
	Not done	Partly done	Well done
Communication			
Information gathering			
Elicited responses using appropriate questions	0	5 (45)	6 (55)
Clarified information by repeating	4 (36)	5 (45)	2 (18)
Allowed patient to talk without interrupting	0	0	11 (100)
Relationship development			
Communicated concern, intention to help	0	4 (36)	7 (63)
Non-verbal behavior enriched communication	0	1 (9)	10 (91)
Acknowledged emotions appropriately	5 (45)	2 (18)	4 (36)
Was accepting, non-judgmental	0	6 (55)	5 (45)
Used words patient understood, explained jargon	2 (18)	3 (27)	6 (55)
Patient education			
Educated patient on Hep B in a culturally sensitive manner	0	6 (55)	5 (45)
Explained risks of transmission: unsafe contact and safe contact	0	4 (36)	7 (63)
Discussed general health education	2 (18)	9 (82)	0
Assessment			
Evaluated reading skills in a compassionate and culturally sensitive manner	11 (100)	0	0
Evaluated patient's understanding of risks of transmission prior to explaining unsafe contact and safe contact	5 (45)	6 (55)	0
Treatment plan			
Clearly discussed next step of follow up plan	1 (9)	9 (82)	1 (9)

Table 2 Apologizing for a complication *n* (%)

Competency areas and specific skills	Distribution of responses *n* = 11		
	Not done	Partly done	Well done
Patient activation			
Helped patient understand the cause of health condition	0	3 (27)	8 (73)
Helped patient understand the different available treatment options	0	3 (27)	8 (73)
Helped patient feel able to follow the recommendations or take the next steps	0	4 (36)	7 (63)
Accountability			
Disclosed complication directly	0	0	11 (100)
Fully explained the complication	0	0	11 (100)
Personally apologized for this complication	7 (63)	0	4 (36)
Took responsibility for situation and recovery	7 (63)	0	4 (36)
Delivering bad news			
Gave opportunity for patient to respond emotionally	0	0	11 (100)
Responded to patient's emotions.	0	0	11 (100)
Shared decision making			
Explored patient's beliefs, values, and preferences	0	5 (45)	6 (55)
Engaged patient in the decision making process	0	1 (9)	10 (91)
Allowed for explicitly deferred decision making	0	4 (36)	7 (63)
Assessment			
Reassured patient that care is appropriately supervised and executed, established trust	8 (73)	a	3 (27)
Elicited understanding that patient mistrusts system, negotiated trust in future quality of care	10 (91)	a	1 (9)

[a]Scored by faculty observer as performed or not performed.

therapy. The goals of the GI fellow were the following: recognize the patient's strong religious beliefs, negotiate her belief system into an agreeable clinical plan of care, and ensure that the patient is amenable to following through with the treatment plan.

Evaluation

Each of the eleven GI fellows participated in all three scenarios detailed above. All OSCE stations were videotaped and observed live by faculty through the use of video media in the NYU School of Medicine Simulation Center for the Health Sciences. The fellows were allotted 15 min for each encounter with the standardized patient. Immediately following each case, feedback was provided to the fellow by the faculty observer and the SP. Data, in the form of checklists and questionnaires were collected from the fellow to specifically address cultural competency as it pertained to each of the given cases. Checklists were created to provide SPs and faculty observers with specific criteria to rate the fellows' performance. The fellows were rated with the use of a three-point scale for each assessed task: (1) "not done" (the fellow did not perform the task); (2) "partly done" (the fellow attempted to perform the task, but was unsuccessful); and (3) "well done" (the fellow addressed and performed the task successfully).

Post-OSCE

After the case scenarios were completed, a debriefing session involving each training program's respective fellows and the faculty observers was held in order for the fellows to provide feedback about the OSCE itself and its relevance as an education tool.

RESULTS

Four faculty members from two academic institutions and eleven fellows from four GI training programs participated. All participants were first-year GI fellows. Four fellows from NYU School of Medicine participated; three from the Icahn School of Medicine at Mount Sinai's training program, and two each from St. Luke's-Roosevelt Hospital Center and Lenox Hill Hospital. Table 1 shows a detailed breakdown of the performance of the fellows in each of the categories assessed by the SP and the faculty observers in the Health literacy Case, as well as the results of the post-OSCE computer-based questionnaire completed by the fellows. Tables 2 and 3 show the results from the second and third cases, respectively.

In the Health Literacy Case, 18% of participants (2/11 fellows) recognized that the patient had below-

Table 3 Breaking bad news *n* (%)

Competency areas and specific skills	Distribution of responses *n* = 11		
	Not done	Partly done	Well done
Patient education			
Gave results of colonoscopy effectively	3 (27)	7 (63)	1 (9)
Explained procedures already done during colonoscopy	0	8 (73)	3 (27)
Explained next necessary steps, how results fit into longer term plans	0	7 (63)	4 (36)
Checked patient's understanding of treatment options	4 (36)	7 (63)	0
Delivering bad news			
Physically set the tone for receiving bad news	3 (27)	8 (73)	0
Assessed your readiness to receive news, gave warning shot	4 (36)	7 (63)	0
Gave opportunity for patient to respond emotionally	1 (9)	7 (63)	3 (27)
Responded to patient's emotions.	1 (9)	7 (63)	3 (27)
Accountability			
Conveyed accountability, assured appropriate treatment and follow up	1 (9)	5 (45)	5 (45)
Shared decision making			
Explored patient's beliefs, values, and preferences	1 (9)	7 (63)	3 (27)
Engaged patient in the decision making process	3 (27)	6 (55)	2 (18)
Allowed for explicitly deferred decision making	1 (9)	6 (55)	4 (36)
Assessment			
Explained and apologized for complication	3 (27)	8 (73)	0
Engaged patient in a discussion about next steps	0	7 (63)	4 (36)
Elicited understanding that patient has different beliefs, negotiated common goals	4 (36)	a	7 (63)

[a]Scored by faculty observer as performed or not performed.

basic health literacy. Below-basic health literacy is defined as the ability to perform only simple and concrete literacy activities (a commonly cited example: the ability to read a set of short instructions, and identify what is permissible to drink before a medical test)[9]. None (0/11) of the fellows evaluated the SP's reading skills. Though none of the fellows thoroughly discussed the SP's general health education and assessed her level of health literacy, nearly 82% (9/11) partly performed the task, while the remaining 18% did not perform this task at all. What was done partly well by more than half of the participants (6/11) and done well by 5/11 of the fellows was the education of the SP on hepatitis B virus in a culturally sensitive manner. Despite the fellows' overall deficient performance on this OSCE station, nearly 73% (8/11) of fellows reported receiving some type of training during their medical school or post-graduate training in patient health literacy.

In the Apologizing for a Complication Case, 4/11 (36%) participants personally apologized for the complication and took responsibility for the situation. Seventy-three percent failed to reassure the patient that the quality of care was appropriately supervised and executed. Finally, only 1/11 (9%) participants recognized that the SP harbored a mistrust of the medical care system. Similar to the previous case, nearly 73% (8/11) of fellows reported receiving some type of training during their medical school or post-graduate training in disclosing medical errors.

With the Breaking Bad News Case, 100% of the fellows agreed that this was the most challenging OSCE of the three scenarios. We found that 2/11 fellows (18%) engaged the SP in the decision-making process. According to the SP's rating, only 27% (3/11 fellows) explored the patient's beliefs, values, and preferences to recognize that she possessed strong religious and fatalistic beliefs. However, faculty observers reported that 63% of fellows effectively elicited understanding that the SP had different beliefs and used this understanding to negotiate a common goal. Only 18% of participants (2/11 fellows) were able to gain the patient's trust and have her agree to pursue further medical care.

DISCUSSION

Cultural competency is central to an effective physician-patient relationship, but teaching its tenets has proven to be a challenge[10]. This skill set has historically been taught implicitly, through observation of faculty and mentors, and by means of self-reflection. Though this hidden curriculum prepares trainees for some aspects of culturally-competent care, it is not without flaws[11]. Current curricula are non-standardized, informal, and sometimes unavailable, leading to a growing interest in teaching cultural competency.

Substantial effort has been afforded to identify and eliminate cultural barriers that impede effective cross-cultural care[12,13]. It is understood that physicians cannot apply a "one-size-fits-all" approach to patient care, especially as our patient population grows increasingly diverse. There is a need for practicums that accurately grasp the educational deficiencies in cultural competency in medical education training. The goal of this OSCE is to fill this void.

The OSCE is a validated tool for measuring performance in various aspects of patient care, particularly with the more difficult-to-measure core competencies[14]. This project is the first of its kind to formally assess and measure gastroenterology fellows' ability to approach challenging clinical scenarios with an attuned understanding and practice of cultural competency in a standardized fashion.

It has long been cited that the lack of culturally-conscious medical care plays a pivotal role in health and health care disparities, and that there is a need to incorporate cultural awareness into the academic setting[13]. Previous, now abandoned, efforts in cultural competency education employed a "categorical approach". The major pitfall of this approach is the concern for stereotyping care for patients based on

socio-cultural backgrounds. The fear has been that this type of educational method assumes cultural "norms" and oversimplifications, thereby sacrificing the physician-patient relationship[2,10]. An alternative approach has since been embraced. By recognizing culturally competency as a skill set, health care providers can effectively deliver care to those of diverse backgrounds in an individualized and more culturally-aware manner[12]. This project systematically assessed and measured areas of strength and weakness in competencies deemed necessary by the ACGME. Our intent is to inform future training curricula for fellows, so that they may provide meaningful cross-cultural care after training.

The literature shows that despite the perceived importance to teach and deliver cross-cultural care, there is little clinical time allotted to honing this skill. Moreover, a large majority of trainees felt unprepared for such challenges[15,16]. In 2008, Lopez *et al*[17] conducted a survey of 2047 residents from seven different specialties and 563 residency programs to determine whether resident physicians' socio-cultural characteristics influence self-perceived preparedness and skill in delivering cross-cultural care. With training received during medical school and/or residency controlled for, the most important factor associated with improved perceived skill level in practices believed to be of use in treating a culturally-diverse patient population was cross-cultural skills training during residency (OR: 1.71-4.22). Our project is a novel and formal assessment of trainees' cross-cultural skills at the fellowship level. The three areas of cultural competency that we chose to focus on included health literacy, disclosing and apologizing for a complication in a patient who harbors mistrust in the health care system, and bearing unfavorable medical information to a patient with a strong religious belief system. The Health Literacy station revealed that few fellows were able to identify the patient as having below-basic health literacy[18], while none of the eleven fellows explored her general health knowledge in order to effectively gauge how best to educate her about her new diagnosis of hepatitis B virus. When the nuances of patient understanding are left unrecognized or unacknowledged by the physician, patient adherence becomes less likely. With the Apologizing for a Complication Case, the majority of the fellows failed to formally apologize or take responsibility for the complication. Moreover, only one fellow was successful in identifying the patient's mistrust in the health care system, which inherently compromised the physician-patient relationship. In recognizing and confronting the grounds for mistrust in a non-threatening, honest manner, effective communication between clinician and patient is far more likely. Finally, the Breaking Bad News station was equally informative. The majority of fellows failed to include the patient in the decision-making process for the next step in management, and only two participants elicited a trustful agreement with the SP.

Our performance-improvement program is not without limitations. Though four different institutions in New York City participated, the number of fellows in this pilot program was small. A longitudinal assessment of cultural competency as a skill set has not yet been performed, and so the utility of assessment with an OSCE remains to be studied. Finally, the Breaking Bad News station illustrated that there is a discrepancy between the standardized patient and faculty observers' perception of fellows' abilities. Therefore, there may be a role for cultural competency training for faculty who are involved in teaching fellows. Expanding the employment of the OSCE into the GI fellowship curriculum for reinforcement of such competencies is an eventual goal that has already shown to be of benefit in this cross-institution initiative, but should be explored further. Awareness and formalized assessment of deficiencies in cultural competency with OSCEs is an important step in developing the appropriate skills for serving a diverse patient population.

With the advent of the ACGME's Next Accreditation System (NAS), it is incumbent upon training programs to evaluate the impact of their training methods on educational outcomes. This is accomplished by assessing trainees based on a number of important outcome-based milestones incorporated within the six domains of physician competence. The scenarios tested in the OSCE mostly directly reflect milestones within the professionalism [Responds to each patient's unique characteristics and needs (PROF3)] and interpersonal communication [Communicates effectively with patients and caregivers (ICS1)] competencies. Implementation of these cultural competence OSCEs into the GI fellowship program curriculum serves a dual purpose. Not only do such OSCEs allow fellowship programs to assess GI fellows according to these milestones during the educational event, but they also permits us to examine the longitudinal impact on the fellows' practice, permitting further assessment of a practice-based learning and improvement milestone [Learns and Improves *via* feedback (PBLI1)]. Therefore, the next step will be to evaluate the trainees' performance in ongoing clinical care to assess the impact of institution of the OSCE on their continued use of cultural competence. In this way, we can be assured that we are meeting our goals of training professional fellows who excel at interpersonal communication in a culturally-competent manner.

OSCEs serve as a validated tool to assess the performance of training physicians' ability to carry out complex clinical skills. The ability to provide cross-cultural care encompasses two of the six core competencies outlined by the ACGME, namely interpersonal skills and communication, and professionalism. This program is novel in its employment of the OSCE to formally test and measure cultural competency as a

vital skill set at the fellowship level. This educational tool informs fellowship curricula, and can be extended to academic faculty as well. More study is necessary to assess why and to what degree such deficiencies in cross-cultural care and educational training exist, and if the implementation of the OSCE as an educational tool improves culturally-competent patient care in the long-term.

COMMENTS

Background

Cultural competency is recognized as an essential component of a productive physician-patient relationship. Despite this recognition, our ability to effectively assess and teach these complex communication and interpersonal skills to medical trainees has not been formalized or standardized, particularly at the fellowship training level.

Research frontiers

Objective structured clinical examinations, or OSCEs, have been validated as a means of assessing complex skill sets in a measurable, reproducible context. Its novel application in cultural competency training at the fellowship level satisfies many of the core competencies set forth by the Accreditation Council for Graduate Medical Education (ACGME).

Innovations and breakthroughs

This experiential learning endeavor for the explicit purpose of both assessing and teaching cultural competency at the fellowship level is the first of its kind. OSCEs have shown to be a wonderful tool to teach complex skill sets. The use of the OSCE during a training physician's advanced stage of formal training works to build upon and potentially reinforce any cultural competency training he or she has been exposed to through both his/her formal and hidden curricula during medical school and residency.

Applications

An eventual result of such an OSCE is to inform further medical curricula in the inclusion of such experiential learning that is centered on cultural competency.

Peer-review

For assessing the performance of training physicians' ability to carry out complex clinical skills, authors designed an OSCE on specific criteria. And the authors also addressed it was vital to providing culturally-competent care.

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ORIGINAL ARTICLE

Observational Study

Snapshot of an integrated psychosocial gastroenterology service

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Abstract

AIM: To characterize the patients utilizing a gastroenterology behavioral medicine service and examine the effect of treatment on health care utilization.

METHODS: Patients were referred by their gastroenterologists for psychological treatment during a 15 mo period. Patients seen for an intake with a psychologist completed the Brief Symptom Inventory (BSI) and a checklist of psychosocial concerns. A subset of patients with functional bowel disorders also completed a disease specific quality of life measure. Chart review was conducted to obtain information on type and frequency of sessions with the psychologist, the number of outpatient gastroenterology visits, and number of gastroenterology-related medical procedures during the 6 mo following psychological intake.

RESULTS: Of 259 patients referred for treatment, 118 (46%) completed an intake with a psychologist. Diagnoses included: irritable bowel syndrome (42%), functional dyspepsia (20%), inflammatory bowel diseases (20%), esophageal symptoms (10%), and "other" (8%). Demographic variables and disease type did not differentiate between those who did and did not schedule an intake. Mean *t*-scores for the BSI global score index and the depression, anxiety, and somatization subscales fell below the cutoff for clinical significance ($t = 63$). Treatments were predominantly gut-directed hypnosis (48%) and cognitive behavioral therapy (44%). Average length of treatment was 4 sessions. Among functional gastrointestinal (GI) patients, those patients who initiated treatment received significantly fewer GI-related medical procedures during the 6 mo following the referral than patients who did not schedule an intake [t (197) = 2.69, $P < 0.01$].

CONCLUSION: Patients are receptive to psychological interventions for GI conditions and there is preliminary evidence that treatment can decrease health-care utilization among patients with functional GI conditions.

Key words: Psychological treatment; Irritable bowel syndrome; Functional gastrointestinal disorders; Hypnosis; Cognitive-behavioral therapy

Core tip: Psychological interventions are effective treatment options for many chronic gastrointestinal conditions, particularly functional bowel disorders. However, psychological care has not been well integrated into standard clinical practice for gastrointestinal disorders. The aim of the current study was to examine the feasibility and acceptability of offering psychological services to patients in an outpatient gastroenterology practice and the potential impact of treatment on health care utilization.

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INTRODUCTION

Chronic gastrointestinal (GI) problems account for a significant proportion of physician workload in outpatient gastroenterology practices. Inflammatory bowel diseases (IBD) and functional bowel disorders jointly account for more than half of the patients seen by gastroenterologists[1]. The cost of managing these conditions is high. The estimated direct cost of irritable bowel syndrome (IBS) alone is estimated to be 1.3 billion in the United States and is similar to other chronic medical conditions such as asthma, hypertension, and congestive heart failure[2,3]. In addition, the psychosocial impact of chronic digestive symptoms is significant. Patients with both functional (*e.g.*, IBS) and organic GI conditions (*e.g.*, IBD) experience impaired quality of life (QOL) and increased rates of psychological distress[4,5].

Traditional medical treatments are often inadequate for managing functional gastrointestinal conditions, particularly for patients with moderate to severe symptoms[6]. As a result these patients often pursue alternative treatment options. In fact more than 50% of patients with IBS and IBD turn to complementary and alternative medicine treatments and nearly 1/3 of patients with IBD express interest in psychological treatment[7-9].

Psychological interventions are now well-established, effective treatments that can complement usual medical care for many chronic GI conditions. Cognitive behavioral therapy and gut-directed hypnosis are empirically supported interventions for IBS and the American Gastroenterological Association recommends psychological treatments for moderate to severe patients[10-12]. In addition to IBS, gut-directed hypnotherapy has been demonstrated as an effective treatment for functional dyspepsia, non-cardiac chest pain, and acid reflux[13-15]. Less is known about the benefits of stress management and behavioral interventions for IBD. However, emerging evidence indicates that psychological interventions are beneficial for improving mood, QOL, and even prolonging remission in IBD patients[16,17].

Many of these positive outcomes can be attributed to patients' increased self-efficacy to manage and cope with their digestive condition, thereby reducing burden on medical providers[18]. There is some empirical evidence that psychological treatments can decrease health care utilization. For example, relaxation training contributed to less frequent doctor visits among IBS patients and psychological treatment was associated with reduced health care costs in patients with IBS[19,20].

Despite the documented benefits of psychological interventions for GI conditions, these treatments have not been well integrated into gastroenterology practices and are not easily accessible to patients. Many barriers have been suggested, including concerns about reimbursement for behavioral services, referrals only being made for patients with significant psychological symptoms, and the misconception that patients are not receptive to psychological interventions[11]. Palsson and Whitehead recently published a primer for gastroenterologists, encouraging providers to consider referring patients with functional GI conditions for psychological treatment and recommendations on making a successful referral[6]. Their article is an important step in explaining how these treatments can become more integrated into the care of patients with chronic GI conditions. The purpose of the current study is to demonstrate how this integration can occur in a clinical context. This is the first study that we are aware of to evaluate the acceptability and feasibility of offering behavioral treatment as part of routine clinical practice within a gastroenterology clinic.

The goal of the current study was to characterize an integrated psychological gastroenterology service and examine impact of this service on health-care utilization. Specific aims were to: (1) examine the number of patients referred and proportion of patients that engaged in psychological treatment for their GI condition; (2) determine any demographic or disease related variables that might influence the likelihood of a patient pursuing treatment; (3) characterize the psychosocial functioning of patients pursuing behavioral treatment; and (4) among patients with functional GI conditions, evaluate the impact of receiving behavioral medicine services on health care utilization (*i.e.*, physician office visits, medical procedures).

MATERIALS AND METHODS

Study patients

This is a retrospective cross-sectional study of patients with chronic GI conditions referred to a GI health psychology service during a 15-mo period from

2010-2011 at our outpatient faculty practice group at Feinberg School of Medicine, Northwestern University. All referrals were made by gastroenterologists within our outpatient practice. Referrals were not accepted from outside providers. Patients had undergone a thorough GI work-up prior to being referred for psychological services. Table 1 provides a list of criteria that we recommend physicians in our practice follow to guide them in referring appropriate patients. Gastroenterologists referred patients by explaining the rationale for psychological treatment and providing patients with a brochure with further details about the services. The gastroenterologist also placed a formal referral through our electronic medical record system that is routed to a patient-liaison who then contacts the patient. This initial phone call provides the patient with opportunity to ask additional questions about behavioral medicine services, schedule the initial appointment, and review insurance coverage for the visit. Any patient referred for management of a chronic gastrointestinal condition during this 15-mo period is included in our sample, including patients who did not respond to the referral.

Table 1 Guidelines for appropriate referrals for health psychology services

Appropriate referrals
Patients with moderate to severe functional symptoms who have not responded to medical management (Palsson and Whitehead, 2013)
Stress or emotional factors are exacerbating gastrointestinal (GI) symptoms
Any patient interested in non-pharmacological treatment of functional GI symptoms
Patients newly diagnosed with chronic GI illness (*e.g.*, crohn's disease; ulcerative colitis)
Any patient needing assistance coping with chronic, uncomfortable GI symptoms
Inappropriate referrals
Patients with significant psychological symptoms that are independent of the GI condition
Current severe psychiatric symptoms (suicidal ideation, psychotic disorder, obsessive-compulsive disorder)
Active eating disorder
Low insight into the role of stress on his/her GI condition
Poor motivation to engage in psychological treatment

Measures

The following psychosocial questionnaires were completed by patients at their intake visit with the psychologist as standard clinical practice. A subset of the sample (*i.e.*, patients with functional bowel disorders; $n = 43$) also completed a QOL measure.

Demographics and psychosocial history: A short demographic and psychosocial checklist was completed by each patient, which includes basic demographic data (*e.g.*, age, gender, marital status, education) as well as a checklist of current and past psychosocial concerns (PSC). The checklist included 36 items of common psychological difficulties (*e.g.*, depression, anxiety, difficulty managing stress, history of abuse, anger). Patients checked "yes" if they had ever experienced the difficulty. Patients also indicated to what extent they believed that stress impacted the course and treatment of their GI condition, a good marker of psychological insight and appropriateness for behavioral intervention.

Brief symptom inventory: BSI is an 18-item questionnaire used to assess psychological distress[21]. The BSI yields a total (general distress) T-score and 3 subscale T-scores for depression, anxiety, and somatization. Higher scores represent greater distress. The cutoff for clinical significance for each scale is $t = 63$. The BSI has good internal reliability (0.71-0.85) and test-retest reliability (0.68-0.91).

IBS-QOL questionnaire: IBS-QOL questionnaire is a 34-item measure designed to assess the impact of bowel habits on overall functioning and QOL for people with IBS or related functional bowel disorders[22]. Each item has a 5-point response scale (1 to 5). Item scores were reversed and then summed to derive an overall QOL index and 8 subscales (dysphoria, interference with activity, body image, health worry, food avoidance, social reactions, sexual activity, and relationships). Scores were transformed to a 1 to 100 scale ranging from 0 (poor QOL) to 100 (maximum QOL).

Retrospective chart review

Chart review was conducted on all patients referred to our service irrespective of following through with a behavioral medicine intake and included: medical diagnosis, number of physician office visits during the 6 mo following the behavioral medicine referral (including emergency room visits or primary care physician visits related to their condition) and number of diagnostic procedures related to their gastrointestinal condition in the 6 mo following the referral. Diagnostic procedures included blood work, stool studies, breath testing, imaging, and endoscopy procedures. Additionally, the number and type of treatment sessions with a psychologist was collected for patients participating in BMed treatment.

Statistical analysis

Statistical analysis were completed using SPSS 18.0 for Windows (SPSS Inc., Chicago IL, United States). Statistical significance was set at $P < 0.05$ for all analyses. Demographics and psychosocial characteristics of the sample were completed computing frequencies, means, standard deviations, and percentages where applicable. We used logistic regression to examine demographic and disease variables as predictors of whether or not patients initiated treatment. Logistic regression was also used

Table 2 Demographics based on response to referral

	Patients seen for an initial intake (n = 118)	Patients not seen for an intake (n = 141)
Demographics		
Average age	40	38
Gender (% female)	71	79
Disease type		
IBD	22	29
Functional bowel	49	59
Dyspepsia	23	30
Esophageal	15	11
Other	9	12

Patients seen for an initial intake: Includes all patients evaluated by a psychologist, including both functional GI disorders as well as other disease groups; Patients not seen for an intake: Includes all patients that were referred for psychological services by their gastroenterologist, but did not meet with a psychologist; "Other" disease group: Chronic pancreatitis, celiac disease, non-cardiac chest pain, rumination syndrome, functional vomiting, gastroparesis, eosinophilic disease, and aerophagia. Diagnosis Type refers to the patients diagnosed with each disease type. IBD: Inflammatory bowel diseases; GI: Gastrointestinal.

to examine differences in HCU among patients who did and did not engage in BMed treatment.

RESULTS

Demographics

A total of 259 internal referrals for psychological treatment were identified from February, 2010 through May, 2011. Diagnoses included IBS (42%), functional dyspepsia (20%), IBD (20%), esophageal symptoms (10%), and "other" (8%; *e.g.*, chronic pancreatitis, non-cardiac chest pain, rumination syndrome, functional vomiting, gastroparesis, aerophagia). The majority of referrals (76.8%; n = 199) were for patients diagnosed with some type of functional GI condition (*e.g.*, IBS, functional abdominal pain, functional dyspepsia, functional vomiting, esophageal hypersensitivity). Average age was 38 (range: 19-88) and 76% were female. Table 2 provides a breakdown of demographic data for all patients referred based on whether or not the patient met with a psychologist for an initial visit.

Nearly half of patients referred for treatment scheduled an intake with a psychologist (n = 118) and 113 of these patients completed intake forms. The following demographics were obtained from the intake forms of these 113 patients: marital status was predominantly single, divorced, or widowed (61%); patients were predominantly college educated (73%); 95% indicated that stress has a moderate or severe impact on the course or treatment of their GI condition.

Feasibility and acceptability

Of the 259 patients referred for treatment, 118 (46%) met with a psychologist for an intake. Approximately one-third (n = 87; 34%) engaged in ongoing psychological treatment with a therapist. Treatments were predominantly gut-directed hypnosis (48%) and cognitive behavioral therapy (CBT, 44%). Mean length of treatment was 4 sessions (SD = 4.32, range: 1-21).

QOL

Disease-specific QOL was obtained from a subset of patients who were diagnosed with a functional bowel disorders using the IBS-QOL (n = 43). Mean IBS-QOL was 58.94. Food avoidance (38.6) was the most impaired subscale domain, followed by dysphoria (52.2), interference with activity (56.8) and health worry (58.8). These symptom reports are comparable to scores reported in the IBS literature[22,23].

Psychosocial characteristics

Frequency of psychosocial concerns and mean BSI scores were calculated on the entire sample of patients that completed intake forms with the psychologist (n = 113). The most frequently reported PSC included anxiety (75%), worry (63%), and difficulty managing stress (61%). Mean t-scores were calculated for the BSI total score and individual subscales. T-scores for the total score (t= 51.12), depression (t = 51.34), anxiety (t = 51.28), and somatization (t = 50.04) scales all fell below the cutoff for clinical significance (t = 63), indicating that on average these patients did not report clinically significant mood difficulties.

Logistic regression analyses were run on the entire sample to determine whether demographic and disease variables predicted whether or not patients followed up on the referral for psychological services. Results indicated that demographic variables (age, gender) and disease type (IBS, functional dyspepsia, IBD, esophageal symptoms, and "other") did not differentiate between those who did and did not pursue treatment (all P > 0.13).

Health care utilization

Logistic regression was used to examine differences in HCU among patients who did and did not engage in psychological treatment. These analyses were limited to those patients diagnosed with some type of functional GI disorder (n = 199). Among functional GI patients (n = 199), those patients who initiated treatment received significantly fewer GI-related medical procedures during the 6 mo following the referral than patients who did not [t (197) = 2.69, P < 0.01], with those in treatment receiving fewer procedures (0.38 *vs* 0.79). There was no difference in the number of physician office visits (0.43 *vs* 0.54).

DISCUSSION

Chronic GI disorders cause significant quality of life impairment and account for a significant pro-

Table 3 Myths and misconceptions of psychological treatments for gastrointestinal conditions

Myth	Fact
Only patients with significant psychological distress will benefit from working with a health psychologist	The majority of patients seen in our clinic do not suffer from a psychological disorder and yet benefit significantly from treatment
Only patients with functional GI conditions are appropriate for referral to psychologist	Patients with a wide-range of GI conditions can benefit (crohn's, ulcerative colitis, chronic pancreatitis, GERD); 20% of patients in this study had IBD
Psychological treatments are expensive	Psychological treatments are often covered by insurance and are associated with reduced long-term health-care costs.
Psychological treatment requires significant time commitment	Many patients can benefit in as few as 4 sessions

GI: Gastrointestinal; IBD: Inflammatory bowel diseases; GERD: Gastroesophageal reflux disease.

portion of health care utilization[24]. There is strong empirical evidence that psychological treatments for functional GI disorders are beneficial; however, the implementation of these treatments in a clinical setting has lagged behind. The results of the present study indicate that it is feasible to implement these treatments in an outpatient GI clinic and that many of the barriers to treatment that have been suggested in the past can be overcome[11]. Table 3 provides a list of common myths about psychological treatments for GI patients and corresponding facts based on findings of this study and our experience running an integrated psychosocial GI service.

Our findings suggest that many patients are in fact receptive to and interested in psychological treatment. Over 200 patients were referred for psychological treatment within a 15-mo time span and nearly half of these patients met with a psychologist. Historically, referrals for psychological treatments are only considered for patients who are significantly distressed. Our experience has been that even patients who are functioning quite well emotionally can still benefit from these treatments. The majority of the patients in the current study identified stress as a contributing factor to their GI condition, but they were not suffering from a psychological disorder. These findings are contrary to the perception that only highly anxious or depressed patients will benefit or be interested in meeting with a psychologist.

It is notable that patients with a wide range of GI conditions utilized behavioral treatments. Traditionally, psychological referrals are only considered for patients with functional conditions. However, 20% of the referrals made in this study were for patients with inflammatory bowel disease and patients with various organic GI conditions are commonly seen by the health psychologist in our clinic (*e.g.*, celiac sprue, pancreatitis, eosinophilic gastrointestinal diseases). Our experience has been that IBD patients in particular are eager to work with a GI health psychologist and this is consistent with research findings[9]. Furthermore, patient demographics did not influence whether or not patients pursued health psychology services. This should encourage gastroenterologists to consider referring a wide range of patients and not assume that certain demographic groups (*e.g.*, elderly) would not be interested.

Our findings are consistent with past research indicating that psychological interventions can reduce healthcare burden. For example, Creed *et al*[19] found that psychological intervention, but not antidepressant medication, was associated with reduced health care costs, largely due to fewer gastroenterology follow-up visits. Similarly, our results indicate that functional GI patients who participate in psychological treatment are less likely to need further medical procedures for their GI condition. Although psychological treatments may require greater time investment from the patient initially, there is less long-term medical follow-up needed (outpatient visits, medical procedures, medications); thereby reducing burden on patients and providers. Given the effectiveness of psychological treatments for GI conditions, it is not surprising that patients engaged in these treatments were in less need of medical attention.

Another significant finding from this study is the short-term nature of treatment. The average length of treatment in our study was 4 sessions. These findings are consistent with past research indicating that a significant proportion of IBS patients treated with CBT respond to treatment within 4 wk (referred to as rapid responders) and maintain symptom improvement for at least 3 mo[10]. These findings are contrary to the misconception that psychological treatment requires a long-term time commitment. CBT is designed to be a short-term skills-based treatment that teaches the patient to self-manage their condition. Patients will be more likely to consider psychological treatment if they understand that this is a short-term investment. It is notable that the treating provider in this study is a health psychologist with specific training and experience in gastroenterology. Implementation of psychological treatments for GI conditions requires specialized knowledge and training and these same results might not be achieved by a "general" psychotherapist. Unfortunately there are a limited number of providers with this expertise, making it challenging for physicians to find psychological providers to refer to.

Our clinical experience has been that many of the suggested barriers to offering psychological treatments for gastrointestinal conditions can be

addressed with appropriate integration of these services. By having a provider on-site, offering behavioral medicine services in conjunction with medical treatment, and streamlining referrals, patients are more receptive to health psychology services. Furthermore, effective communication of the referral by the gastroenterologist is important[6]. Concerns have also been raised about financial cost as a barrier to psychological treatment. It is worth noting that health psychologists have specialized billing codes (*i.e.*, health and behavior codes) that allow for behavioral treatment to be billed under the patient's medical insurance. All patients seen for treatment in this study received behavioral intervention through their medical insurance. These billing codes not only make treatments more accessible to patients, but also de-stigmatize treatment by differentiating it from mental health treatment.

Although this study contributes important findings, its limitations should be noted. Our sample was drawn from Northwestern's outpatient gastroenterology clinic, located in downtown Chicago. This patient population is typically well-educated, affluent, and has adequate resources to benefit from psychological treatments. Additional barriers might be present for patients from different geographical areas with more diverse socioeconomic status. This study was also limited by small sample size of various disease groups. An important goal of future research will be to obtain chart review data on a larger portion of patients, collect additional measures of HCU (*e.g.*, clinic phone calls, medication use), and examine barriers to participating in psychological services. Despite these limitations, this study provides insight into the role that psychologists can play in improving care and outcomes of patients with gastrointestinal conditions.

COMMENTS

Background

Chronic gastrointestinal (GI) conditions can cause significant quality of life impairment and account for a significant proportion of health care utilization. Traditional medical treatments are often inadequate for managing these conditions, particularly for patients with moderate to severe functional GI disorders.

Research frontiers

Psychological interventions such as medical hypnosis and cognitive-behavioral therapy (CBT) are empirically supported interventions for a variety of functional GI disorders, such as irritable bowel syndrome. These psychological treatments provide symptom relief in addition to improvements in mood and quality of life. Although there is strong empirical support for the use of psychological treatments with GI patients, the implementation of these interventions in clinical settings has been limited.

Innovations and breakthroughs

This study demonstrates the feasibility of integrating psychological treatment in an outpatient GI clinic. Gastroenterologists are often hesitant to refer patients for psychological treatment due to misconceptions about these interventions and perceived barriers to treatment. The findings of this study indicate that a wide range of patients are receptive to psychological treatment and that these interventions can decrease reliance of patients on medical providers.

Applications

This article provides a model for integrating psychological services into an outpatient GI practice and documents the benefits for both patients and providers of offering integrated psychological care.

Terminology

CBT refers to a type of psychotherapy that is short-term, collaborative, and aimed at addressing symptoms by helping the patient change thinking, behavior, and emotional responses. CBT has been found to be effective for numerous mental and physical health disorders.

Peer-review

This is an interesting study of the feasibility and effect of psychological interventions in the management of common GI disorders. The manuscript is well written, the tables are well organized, and the results are outlined in a very effective manner. This article will attract a significant number of readers, especially among gastroenterologists with specific interest in functional GI disorders.

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ORIGINAL ARTICLE

Observational Study

Measures of patient radiation exposure during endoscopic retrograde cholangiography: Beyond fluoroscopy time

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Abstract

AIM: To determine whether fluoroscope time is a good predictor of patient radiation exposure during endoscopic retrograde cholangiopancreatography.

METHODS: This is a prospective observational study of consecutive patients undergoing endoscopic retrograde cholangiopancreatography in a tertiary care setting. Data related to radiation exposure were collected. The following measures were obtained: Fluoroscopy time (FT), dose area product (DAP) and dose at reference point (DOSERP). Coefficients of determination were calculated to analyze the correlation between FT, DAP and DOSRP. Agreement between FT and DAP/DOSRP was assessed using Bland Altman plots.

RESULTS: Four hundred sixty-three data sets were obtained. Fluoroscopy time average was 7.3 min. Fluoroscopy related radiation accounted for 86% of the total DAP while acquisition films related radiation accounted for 14% of the DAP. For any given FT there are wide ranges of DAP and DOSERP and the variability in both increases as fluoroscopy time increases. The coefficient of determination (R^2) on the non transformed data for DAP and DOSERP versus FT were respectively 0.416 and 0.554. While fluoroscopy use was the largest contributor to patient radiation exposure during endoscopic retrograde cholangiography (ERCP), there is a wide variability in DAP and DOSERP that is not accounted for by FT. DAP and DOSERP increase in variability as FT increases. This translates into poor accuracy of FT in predicting DAP and DOSERP at higher radiation doses.

CONCLUSION: DAP and DOSERP in addition to FT should be adopted as new ERCP quality measures to estimate patient radiation exposure.

Key words: Cholangiopancreatography; Endoscopic retrograde; Fluoroscopy; Radiation; Endoscopy; Standards

Core tip: Endoscopic retrograde cholangiography (ERCP) performance requires endoscopic skills and the use of

fluoroscopy with inherent patient and staff radiation exposure. Current ERCP quality measures do not include any measures of radiation. There has been a suggestion to include fluoroscopy time as a radiation quality measure in ERCP. This article provides data on the strength of correlation between fluoroscopy time and more direct measures of radiation exposure such as dose area product and dose at reference point. It also provides a recommendation to include all three measures as quality measures for ERCP. The article presents important principles to achieve the as low as reasonable achievable radiation doses during ERCP.

Kachaamy T, Harrison E, Pannala R, Pavlicek W, Crowell MD, Faigel DO. Measures of patient radiation exposure during endoscopic retrograde cholangiography: Beyond fluoroscopy time. *World J Gastroenterol* 2015; 21(6): 1900-1906 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i6/1900.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i6.1900

INTRODUCTION

Endoscopic retrograde cholangiography (ERCP) is one the most resource intensive complex endoscopic procedures routinely performed. It requires in addition to endoscopic skills the ability to interpret radiologic images in real time. It is also the one with the highest risk. The American Society for Gastrointestinal Endoscopy (ASGE) and the American College of Gastroenterology (ACG) established a task force in 2006 to create quality metrics for endoscopy including ERCP. The quality measures proposed for ERCP are listed in Table 1 and are designed to represent measurable endpoints indicative of high quality care[1].

The use of fluoroscopy inherent to ERCP results in patients and staff radiation exposure. Since publication of the 2006 guidelines, fluoroscopy time has been proposed as a potential quality metric to add to the original quality measures which did not include a measure of radiation exposure[2]. Radiation exposure in the United States and worldwide have been increasing significantly[3]. Radiation doses to patients and staff during ERCP can be similar to other interventional radiologic procedures[4,5]. While the effects of radiation from one ERCP are unlikely to have any negative effects on patients' health, the cumulative effects of multiple radiologic procedures including ERCPs can be detrimental. A recent study suggested that radiation doses during ERCP may have declined in recent years partly due to better equipment and partly due to the experience gained in ERCP[6]. We believe that measures of radiation exposure during ERCP need to be included as quality indicators. Good measures should be accurate and easily measured. They should be comparable across centers to compare performance and be included in quality improvement projects. They can help endoscopists become more cognizant of their radiation use and in time reduce patient and staff radiation exposure. The ideal measures would also be comparable to measures in other radiologic procedures as we envision a patient specific radiation exposure measure and ways to minimize radiation exposure as one of the future goals of the healthcare system. Fluoroscopy time has been proposed to be used as a quality measure for ERCP, however, fluoroscopy time is but one of several factors that determine radiation exposure, may not be the most accurate surrogate marker and has its limitations. In fact guidelines for patient radiation dose management from the society of interventional radiology recommend that fluoroscopy time be used with caution to monitor patient radiation doses because of poor correlation with other dose metrics)[7]. This article attempts to define an evidence based quality measure of patient radiation exposure specific to ERCP.

Table 1 Proposed quality Indicators for endoscopic retrograde cholangiography

Quality indicator
1 Appropriate indication
2 Informed consent
3 Assessment of procedural difficulty
4 Prophylactic antibiotics
5 Cannulation rates
Desired duct
Use of precut
6 Extraction of common bile duct stones
7 Biliary stent placement
8 Complete documentation
9 Complication rates: pancreatitis, bleeding, perforation, and cholangitis

When X-ray energy is absorbed by tissue an electrical charge is produced. In the international system of units (SI) this is measured in Grays. 1 Gray = 1 J/kg. Because different tissues absorb radiation differently the energy produced is tissue dependant. The radiation dose absorbed in humans is measured in tems (radiation equivalent in men) the unit of which in the SI is Sieverts (Sv)[8]. Ionization can cause DNA damage. There are many different forms of radiation related injuries including stochastic and deterministic injuries. In stochastic the probability of an event is related to the amount of exposure but the severity is not; such is the case in cancer induction. Deterministic injuries occur after a certain threshold is reached; an example would be skin related burns[6]. In stochastic injuries there is no amount of radiation which does not lead to possible injury and thus the concept of linear-no-threshold model of radiation exposure. A consequence of this model is the evolution of the concept of using radiation doses as low as reasonably achievable to perform the task or study (ALARA principle). A simplistic estimate of radiation risk from epidemiologic studies suggest that a lifetime exposure to 1 Sv increases the cancer risk by 10% and cancer mortality by 5%[9]. For reference

Table 2 Radiation data mean and range

	DAPt	DOSERPt	DAPf	DOSERPf	DAPa	DOSERPa	FLUORO_TIME
Mean	0.0022529	0.28213	0.1269457	0.126946	0.000296	0.001784	7.31
Minimum	0.0000013	0.00004	0.0000013	0.00004	0	0	1.00
Maximum	0.004545	1.92667	0.0042557	0.4832	0.000289	0.0291	2141

a CT scan exposes the patient to around 10 mSv and translates to an increase by one cancer in every 1000 CT scans. There are many measures of radiation exposure that can be used. Dose Area Product (DAP) is the product of the dose absorbed and the area irradiated and is expressed in Gy square cm. It is an estimation of the entire dose of radiation that the patient receives and is thought to correlate the with long term biologic risk from radiation or stochastic injury. Dose at the reference point (DOSERP) is another measure used and is the dose of radiation delivered to a specific point in space which is, unless otherwise specified, along the central ray 15 cm from the isocenter toward the X-ray tube. DOSERP is relevant to skin injury and deterministic injury. Both can be easily measured by detectors installed on the fluoroscopy unit and the results can be made to be automatically included in reports and transmitted to a database. Because of the ease of measurement, fluoroscopy time has been used as a measure of radiation exposure in ERCP. The assumptions are that FT is a good indicator of radiation exposure and their relationship is linear. However, FT is just one of several factors that determine radiation exposure. These factors include acquisition (spot) films, fluoroscopy pulse rate, patient distance from the x-ray tube, use of oblique imaging, magnification and patient body mass index (BMI). In fact, multiple studies of non-GI interventional radiologic procedures have found FT to be a poor predictor of patient radiation doses such as interventional radiology societies caution against relying exclusively on FT as a measure for patient radiation exposure[5,7,10-12].

MATERIALS AND METHODS

A part of an ongoing quality initiative we prospectively collected data from all ERCPs performed in our tertiary care center from January 2012 until June 2013. The following information was obtained: Dose area product (DAP) in milligray meter squared, radiation dose of the reference point in Gray (Gy) DOSERP and fluoroscopy time (FT). DAP and DOSERP data were divided into total, fluoroscopy related and spot films acquisition related: DAPt, DAPf, DAPa, DOSERPt, DOSERPf and DOSERPa respectively. The fluoroscopy unit used was a Siemens unit with the following model and settings: Model - Artis zee multipurpose stand, Software - VC14J, Detector - Flat panel, Pulse per second - 3 PPS for Fluoroscopy, kVp - 125 kVp for fluoro and 120 kVp for spots (maximum) and mA - 800 mA for fluoro (maximum). 0.1 mm Copper filtration was used in addition to the standard filtration. Radiation meters permanently installed on the fluoroscopy unit provided DAP and DOSRP measurements. Scatter plots were generated. Coefficients of variation were determined on both the transformed and non transformed data. Bland Altman plots were obtained. Statistical analysis was performed using SPSS. The study was exempt from review of the institutional because the data was collected without patient identifiers.

All procedures were performed by three experienced therapeutic endoscopists with the possible involvement of a 4th year advanced endoscopy fellow. Procedures were done in the prone position under general anesthesia. Olympus endoscopic equipment and Boston Scientific short wire rapid exchange accessories were used.

RESULTS

Four hundred sixty-three data sets were obtained. ERCPs were performed by three different attendings. A fellow was involved in approximately 60% of the procedures. The radiation data mean and ranges are shown in Table 2. Fluoroscopy time average was 7.3 min. Fluoroscopy related radiation accounted for 86% of the total DAP while acquisition films related radiation accounted for 14% of the DAP. Every acquisition film was equivalent to approximately 15 seconds of fluoroscopy time (data obtained from a sample of the total data).

Scatter plots for DAP and DOSERP as a function of FT are shown in Figure 1. The scatter plot show that for any given FT there are wide ranges of DAP and DOSERP and the variability in both increases as fluoroscopy time increases. This is confirmed by the Bland Altman plots where a significant proportional bias was seen and increased as FT increased (Figure 2). The coefficient of variation (R) for DAP versus fluoroscopy time was 0.645. The coefficient of determination (R^2) on the non transformed data for DAP and DOSERP versus FT were respectively 0.416 and 0.554. A better linear relationship was found using the log transformed data and the coefficient of variation were 0.66 and 0.69. Data on magnification were available on 183 patients. Changes in magnification accounted for only 6% of the variability in DAP and DOSERP.

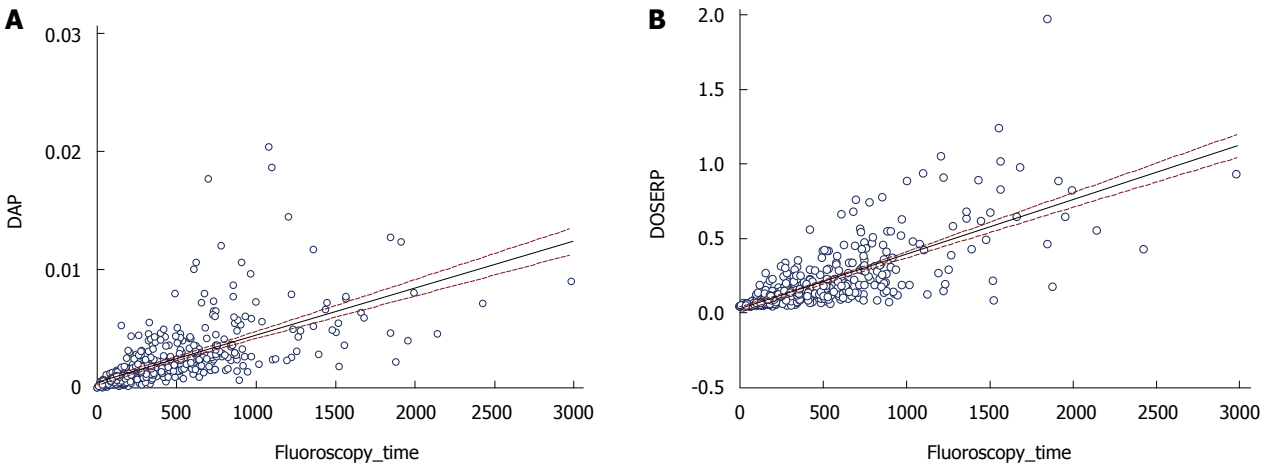

Figure 1 Scatter plot. A: Dose area product and fluoroscopy time; B: Dose at reference point and fluoroscopy time. DAP: Dose area product; DOSRP: Dose at reference point.

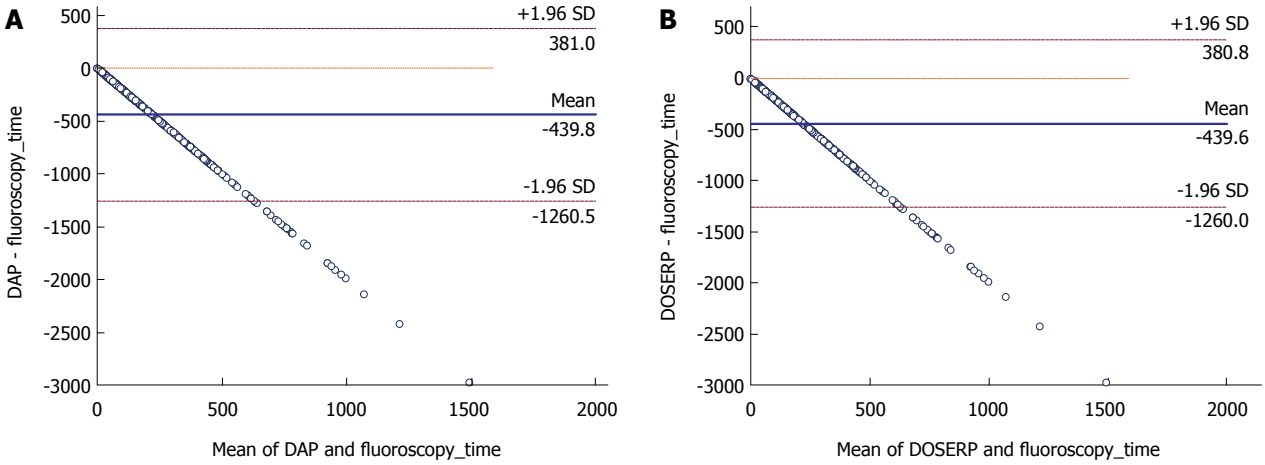

Figure 2 Bland Altman plott. A: Dose area product and fluoroscopy time; B: Dose at reference point and fluoroscopy time. DAP: Dose area product; DOSRP: Dose at reference point.

DISCUSSION

The use of fluoroscopy is inherent to ERCP. Since the quality measures proposed by the joint ASGE/ACG task force there has been proposal to include fluoroscopy time as a additional quality indicator to monitor and improve on patient radiation exposure during ERCP[2] . In addition, most studies which looked at patient radiation exposure during ERCP in the past relied on FT. For example a study which looked at factors associated with increased patient exposure used FT as a measure of radiation exposure[13]. Another study which found that radiation exposure during ERCP was lower when providers with more experience performed the procedure used FT as the outcome measured[14]. Other studies looked at the critical determinant of fluoroscopy duration, the effect of training on fluoroscopy duration and the effect of time limited fluoroscopy[15-17]. One very large study which looked at the experience of the endoscopist and "radiation exposure" in ERCP found that more experienced endoscopist used less fluoroscopy time. These studies assumed that radiation exposure strongly correlated with FT[15]. Prior studies which looked at the relationship between DAP and FT had a small number of patients 20, 73 and 54 patients[4,18,19]. A recent study found no correlation between DAP and FT or total number of films taken[20]. A large study which reported fluoroscopy time, DAP and DOSERP found a strong but "not perfect" with an $r = 0.728$[6]. Our data shows that fluoroscopy use is the largest contributor to patient radiation exposure during ERCP. While there is a good correlation between DAP, DOSERP and FT, there is a wide variability in DAP and DOSERP that is not accounted for by FT. DAP and DOSERP increase in variability as FT increases, and this translates into poor accuracy of FT in predicting DAP and DOSERP where it matters most *i.e.* at higher radiation doses. Thus, while there is a correlation between FT and exposure, the correlation is not accurate resulting in both under and overestimations of radiation dose if FT were relied upon alone.

Figure 3 Good patient positioning: Patient is close to the detector and far from the X-ray tube.

DAP and DOSERP reflect multiple other factors not reflected by FT including the patient size and position, the geometry and setting of the fluoroscopy equipment, collimation, angulation, magnification, total number of acquisition films obtained, and radiation filtration. While some factors such as patient size are not controllable by the endoscopist many are modifiable. Some factors can be modified a priori and for all procedures like the equipment settings including pulse per second and filtration. For example changing the pulse rate on the machine from 15 to 3 can decrease patient radiation doses fivefold. This will not be reflected in FT. Copper filtration will filter radiation to the patient which does not contribute to the quality of images and thus decreasing patient radiation exposure without significantly affecting the quality of the image[21]. Some ways to decrease radiation exposure require meticulous attention at the beginning of the procedure like the patient and detector position (Figure 3).

Others ways to decrease radiation doses need behavior modification during the procedure: For instance using last image save instead of acquisition films and using magnification only when it helps significantly in the task being performed and reverting back to the lowest needed magnification once the higher resolution is no longer needed. That being said, in our study magnification had a small effect on total exposure and we believe that the magnification relationship to total radiation exposure is more complex than the obvious. While there is no doubt that magnification increases the amount of radiation per unit time delivered to a point in space, it may involve a smaller radiation field and thus the effect on DAP is complex. In addition, if magnification allows the faster performance of the needed task it might decrease fluoroscopy time with the end result being less radiation than what would be expected purely from magnification. This might explain why magnification was only responsible for 6% of the variation in our data. We would like to emphasize however that magnification does not always lead to better visualization especially if multiple magnification factors are used as the image can become more blurred at higher magnification. This can happen because magnification can alter the focus of the radiation beam on the detector. Suggestions to help the endoscopist follow the ALARA principles are listed in Table 3.

Table 3 ALARA principles

ALARA principles	
Keep the patient away from the radiation source	Use fluorosave instead of acquisition images
Keep the detector close to the patient	Keep angulation to a minimum
Lower the exposure rate (PPS)	Add 0.1 mm Cu filtration for all protocols
Use lowest needed magnification	Step back during acquisition
Use collimation	Use personal protective equipment
Limit fluoroscopy on-time	Use lead shielding on the fluoroscopy unit

There are additional benefits for using more direct measures of radiation exposure such as DAP and DOSERP over FT. They are comparable among centers and can be used to establish useful benchmarks for quality improvement. They are also comparable to measures obtained during other imaging procedures and interventional radiologic procedure making a patient centered cumulative radiation measure possible. They will also help the endoscopist collaborate with the radiology department to identify ways to decrease patient and staff radiation exposure beyond just looking at FT. DAP and DOSERP have their limitations. For example they both ignore the radiation delivered to the patient as a result of backscatter. While DOSERP is a good estimate of the skin dose delivered to the patient, the best estimate of skin injury would be the peak skin dose (PSD). PSD represents the highest level of radiation that any part of the skin receives. PSD however is very difficult to measure or determine. Both DAP and DOSERP are easily measured and the values collected can be automated making quality improvement projects easier to implement. In addition current FDA guidelines require any new fluoroscopic unit installed in the United States to have the capability of measuring radiation exposure making this type of quality measure eventually possible for all endoscopists using fluoroscopy. The uncertainties in these measures are currently estimated to be + or -50% for DOSERP and +130% to -70% for FT. DAP uncertainty in measurement is in between these values. While none of these measures are highly accurate in determining the exact amount of radiation exposure, FT is the least accurate[20]. For the above reasons, we believe it is time for endoscopists performing ERCP on regular basis to join other fluoroscopy based disciplines in monitoring their patient radiation exposure by incorporating DAP and DOSERP measurements in addition to FT as part of their quality measures.

Patient radiation exposure is increasing in the United States and worldwide. Patient radiation exposure during ERCP can be similar to other interventional radiologic procedures. Quality measures reflecting patient radiation exposure during ERCP are needed. Based on the above data, we recommend adopting DAP and DOSERP in addition to FT as new ERCP quality measures to estimate patient radiation exposure.

COMMENTS

Background

Endoscopic retrograde cholangiography (ERCP) is one of the most complicated gastrointestinal procedures routinely performed. It requires in addition to endoscopic skills the use of fluoroscopy with inherent patient and staff radiation exposure. Quality measures were proposed in 2006 by a joint the American Society of Gastrointestinal endoscopy and the American College of Gastroenterology task force but did not include any measures of radiation. Since the publication of these quality measures fluoroscopy time has been proposed to be added as a measure reflecting patient radiation exposure.

Research frontiers

Studies of fluoroscopy time correlation with patient radiation exposure during ERCP are small. Interventional radiology and cardiology literature suggest that fluoroscopy time is an inaccurate measure of radiation exposure. Well designed large studies looking at ERCP radiation quality measures are lacking.

Innovation and breakthroughs

This is the largest study on radiation measures in ERCP. The findings are contrary to prior small studies which showed that fluoroscopy time is an excellent measure of patient radiation exposure. The findings are consistent with other fluoroscopy based disciplines and recommendation of interventional radiology societies.

Applications

The authors recommend using Dose Area Product and Dose at Reference Point in addition to Fluroroscopy time as measures of patient radiation exposure. These measures will allow creation of ERCP specific radiation benchmarks which can be comparable among centers. They will also allow the possibility of tracking total radiation dose for a given patient across disciplines including diagnostic imaging.

Terminology

Dose area product (DAP): is a surrogate marker of the radiation risk to the tissue irradiated. It is the product of the radiation dose absorbed and the area irradiated expressed in gray cm square. It does not account for the radiation dose cause by scatter. It is easily measured by placing a dosimeter beyond the collimator in a way to intercept the radiation beam. DAP correlates with the risk of stochastic effects such as cancer induction. Dose at reference point (DOSRP): is the dose of radiation delivered to a specific point in space which is, unless otherwise specified, along the central ray 15 cm from the isocenter toward the x-ray tube. It does not include radiation related to backscatter. DOSRP correlates with the risk of deterministic effects such as skin injury. Peak Skin dose: is the highest radiation dose received by any part of the patient skin. This includes radiation from the primary X-ray bean and backscatter. It is difficult to measure but is the best estimate of deterministic effects. Stochastic effects: a radiation effect whose probability of occurrence is related to the amount of exposure but the severity is not; such is the case in cancer induction. Deterministic effects: a radiation effect whose probability occurs after a certain threshold is reached; an example would be skin related burns.

Peer-review

This is a very interesting prospective and descriptive study. The authors have been able to show that fluoroscopy time is not an accurate indirect measurement of the radiation exposure during the ERCP procedure to the patient and medical staff.

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ORIGINAL ARTICLE

Observational Study

Significance of low level infliximab in the absence of anti-infliximab antibodies

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Author contributions: Ungar B, Anafi A and Yanai H analyzed the data; Ungar B drafted the manuscript; Yavzori M, Fudim E and Picard O performed the laboratory research; Kopylov U, Ron Y, Loebstein R, Dotan I and Eliakim R assisted in data acquisition and drafted the manuscript; Ben-Horin S conceived the study, designed the research and drafted the manuscript; all authors finally approved the manuscript.
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Abstract

AIM: To evaluate the prevalence of double negative (DN) sera and the mechanisms responsible for DN status.

METHODS: Sera of inflammatory bowel disease patients treated with infliximab (IFX) were tested for drug/antibodies to infliximab (ATI) trough levels and the proportion of DN results was compared between a commercially available double antigen ELISA (with labeled IFX as the detection antibody) and an anti-lambda ELISA (with anti-human lambda chain detection antibody). Repeat testing with lower than customary serum dilution (1:10) was performed. Patients with DN status were matched with IFX+/ATI- controls and were followed-up for subsequent development of non-transient ATI to investigate if DN status precedes ATI.

RESULTS: Of 67 sera obtained at time of loss of response, only 6/67 (9%) were DN by anti-lambda ELISA compared to 27/67 (40%) with double antigen ELISA ($P < 0.001$, Fisher's Exact test). Of the latter 27 sera, 22% were also DN by anti-lambda ELISA, whereas 44% were actually IFX positive (IFX+ATI-), 30% were ATI positive (IFX-ATI+) and 4% were double positive (IFX+ATI+). Re-testing using a 1:10 dilution converted most DN results into IFX+ and /or ATI+ status. Patients with DN status had shorter survival free of non-transient ATI compared with matched controls (log rank test, $P < 0.001$). In 9/30 (30%) of these patients, non transient ATI occurred before and after the event at which the DN serum was obtained, supporting the view that a DN result may represent a

particular time-point along the two curves of ATI titer rise and infliximab drug level decline.

CONCLUSION: DN status may result from false negative detection of IFX or ATI by double antigen ELISA, suggesting a transitional state of low-level immunogenicity, rather than non-immunological clearance.

Key words: Inflammatory bowel disease; Biological therapy; Infliximab; Immunology; Drug response

Core tip: Among patients who lose response to infliximab (IFX) 10%-60% have low IFX levels in the absence of antibodies to infliximab (ATI) - double negative (DN) status. We explored the prevalence and the mechanisms responsible for DN status. The prevalence of DN sera varied with the assay and dilution used. Patients with DN status had shorter survival free of ATI compared with matched controls ($P < 0.001$). We believe that DN status may result from false negative detection of IFX or ATI by a conventional ELISA assay, suggesting a transitional state of low-level immunogenicity, rather than non-immunological drug clearance.

Ungar B, Anafy A, Yanai H, Ron Y, Yavzori M, Picard O, Fudim E, Loebstein R, Kopylov U, Chowers Y, Dotan I, Eliakim R, Ben-Horin S. Significance of low level infliximab in the absence of anti-infliximab antibodies. *World J Gastroenterol* 2015; 21(6): 1907-1914 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i6/1907.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i6.1907

INTRODUCTION

Infliximab (IFX) is a chimeric mouse - human monoclonal immunoglobulin G1 (IgG1) antibody against tumor necrosis factor α (TNFα). It is effective in inducing and maintaining remission in crohn's disease (CD) and ulcerative colitis (UC)[1-3]. Between 30%-70% of patients who initially respond to IFX subsequently lose their response and experience exacerbation of symptoms, necessitating either dose escalation, switch to another anti-TNF agent, concomitant immunomodulator therapy or surgical intervention[4-6]. Antibodies to infliximab (ATI) develop in approximately 40% of IFX treated patients and correlate with lower IFX trough levels and clinical loss of response (LOR)[7,8]. In 10%-60% of LOR patients, pharmacokinetic tests reveal low IFX trough levels and absence of detectable ATI, designated double negative (DN) status (IFX-/ATI-)[5,9]. Furthermore, several studies, including the SONIC trial, demonstrated that among patients with LOR, the DN status was in fact the more common scenario rather than the expected IFX-/ATI+ status[7,10].

There is a lack of data regarding the mechanisms responsible for the DN status and its consequence. DN status has been attributed to both immune and non-immune clearance of anti-TNF, as well as to technical limitations, such as non-uniform timing of measurement (trough levels are more sensitive than in-between infusions)[5,11]. The uncertainty about the causes and implications of an IFX-/ATI- status makes it hard to establish optimal strategies to prevent and/or manage LOR events in the presence of such a pharmacokinetic situation.

The aims of the present study were to evaluate the frequency and clinical significance of DN status among IFX-treated IBD patients (both in general and at time of LOR) and to investigate the impact of the diagnostic technique on the incidence of this phenomenon.

MATERIALS AND METHODS

Study design and patient population

The study population included IBD patients treated with IFX at the gastroenterology departments of Sheba medical center and the Tel-Aviv Sourasky Medical Center between February 2009 and October 2013, who had available sera stored. All participants provided written informed consent and the ethics committees of the two medical centers approved the study. Pre-infusion sera were obtained and analyzed for trough IFX and ATI levels. Sera of patients whose infusions were delayed for over 2 wk from the scheduled date were excluded.

The study consisted of two separate parts: (1) an analytical part, which targeted differences between assays and technical limitations; and (2) a clinical part, aiming to study the natural history of the DN phenomenon (Figure 1). In the analytical part of the study, IFX and ATI trough levels of patients experiencing LOR were evaluated using two different ELISA assays: double antigen and anti-lambda ELISA. Subsequently, the fraction of IgG4 ATI was measured and compared in a sample of patients with discrepant results between the two ELISA assays to investigate if the conflicting results stemmed from a predominant monovalent IgG4 ATI response. Finally, to investigate the analytical accuracy of the anti-lambda ELISA, this assay was repeated in 45 randomly selected DN sera using a serum dilution of 1:10 (rather than the conventional 1:100 dilution). Patients' sera in this analysis were tested regardless of response status, and sera of healthy volunteers unexposed to IFX served as controls.

The clinical part was a case-control study; cases were patients with IBD who had at least one DN (IFX-ATI-) measurement during routine follow-up (not necessarily at a point of LOR) and controls were IBD patients with positive drug levels without ATI (IFX+ATI-). The starting point of the analysis was defined as the month of the DN event in the

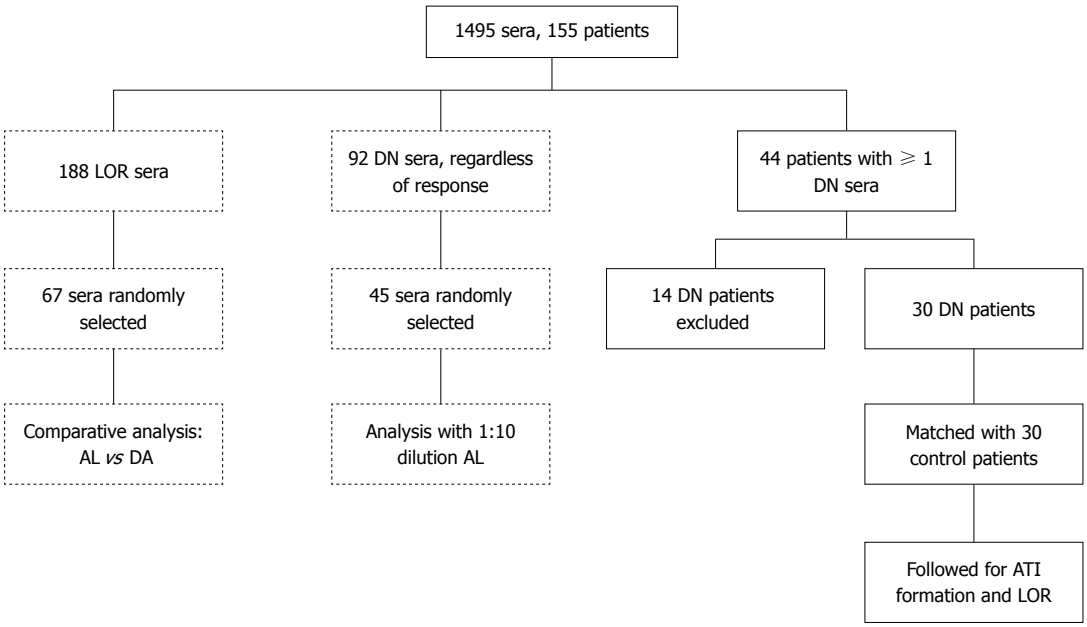

Figure 1 Flow chart of the patients included in the two parts of this study. The analytical part (dashed lines) comprised a comparison of two different assays and of two different serum dilutions; the clinical part (solid lines) followed up, in a case-control study, double negative patients versus patients with adequate infliximab levels for subsequent antibodies to infliximab formation and clinical outcome. DN: Double negative; LOR: Loss of response; AL: Anti-lambda; DA: Double-antigen; IFX: Infliximab; ATI: Antibodies to infliximab.

cases and the matching month in the controls. The pharmacokinetics at the end of follow-up were correlated to clinical outcome. Cases and controls were matched according to the duration of IFX therapy. Patients with unavailable subsequent measurements were excluded. Antibody formation was defined as positive when a patient tested positive for ATI during follow-up on more than two consecutive time points. Transient antibodies were defined as measurable ATI on up to two consecutive infusions, which disappeared on subsequent infusions without any alteration of therapy[8,12,13]. Permanent, non-transient, ATI comprised the primary end point, while transient ATI were disregarded. Clinical response was defined by an improvement in disease activity indexes, the Harvey-Bradshaw index (HBI) and the simple clinical colitis activity index (SCCAI) for CD and UC patients, respectively, coupled with a treating physician's decision to continue IFX therapy without alteration. Clinical response was evaluated on the day of IFX infusion. Secondary LOR was defined as increased disease activity (a rise of > 3 points in HBI score or of > 2 points in SCCAI for CD and UC, respectively) after achieving an appropriate induction response[14-16]. When unavailable, clinical response was determined by the documented physician's global assessment.

Measurement of IFX by TNF-α-blocker-monitoring infliximab drug level ELISA

IFX levels were measured by a commercially available quantitative ELISA, TNF-α-blocker-monitoring (Immundiagnostik, Bensheim, Germany), following the manufacturer's instructions. The assay's detection threshold was IFX > 1 μg/mL.

Measurement of ATI by TNF-α-blocker-ADA double antigen ELISA

ATI levels were measured by a commercially available qualitative TNF-α-blocker-ADA (antibodies against infliximab, Immundiagnostik, Bensheim, Germany), following the manufacturer's instructions. The assay's detection threshold was ATI > 10 AU/mL, which was standardized in our laboratories to 1 AU/mL.

In house determination of IFX levels

A volume of 100 μL of 1:100 diluted serum was added to pre-plated 750 ng/mL TNFα (Peprotech, Rocky Hill, NJ, United States) and incubated for 90 min. Following washing, horseradish peroxidase (HRP) labeled goat anti-human Fc fragment antibody (MP Biomedicals, Solon, OH, United States) at a concentration of 0.62 μg/mL was added for 60 min and reacted with the tetramethylbenzidine (TMB) substrate. The results were then read on an ELISA reader. Quantification of the measured IFX concentration was done by calibration to a standard curve in which exogenous IFX (Schering Plough, NJ, United States) was added at concentrations between 3 and 200 ng/mL. The assay's detection threshold was IFX > 0.6 μg/mL.

Measurement of ATI by anti-human lambda chain detection antibody ELISA

ATI were determined as previously described[11,17]. Briefly, IFX (0.1 mg/mL) was added to pre-plated TNFα (500 ng/mL) in 100 μL wells of ELISA plates

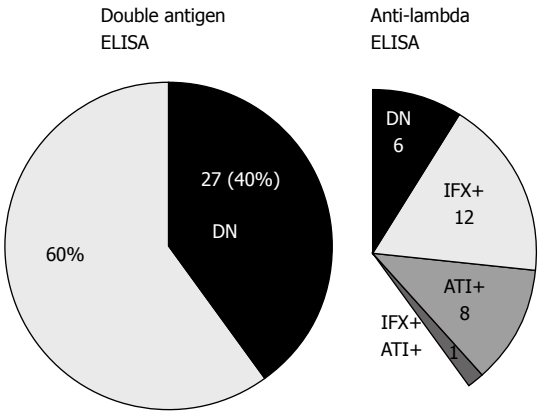

Figure 2 ELISA tests of patients with loss of response. Out of 67 patients with loss of response, 27 (40%) were double negative with double antigen ELISA. Of those, only 6 (9%) were DN using anti-lambda ELISA. LOR: Loss of response; DN: Double negative; IFX: Infliximab; ATI: Antibodies to infliximab.

(Nunc, Roskilde, Denmark). After drying, 100 μL of serum (1:100 dilution) was added and incubated for 90 min at room temperature. Plates were then washed and goat anti-human λ chain HRP-labeled antibody (Sertec, Oxford, United Kingdom) was added at a dilution of 2.5×10^4 for 60 min and reacted with the TMB substrate. The results were read by an ELISA reader EL-800 (Biotek Instruments, Winooski, VT, United States) and expressed as mcg/mL-equivalent (mcg/mL-e) after normalization *vs* results obtained using additions of graded concentrations between 9 and 600 ng/mL of HRP labeled goat anti-human F(ab')2 fragment antibody (MP Biomedicals). The assay's detection threshold was ATI > 2.5 μg/mL-eq.

Measurement of IgG4 fraction of ATI

IFX (0.1 mg/mL) was added to pre-plated TNFα (500 ng/mL) in 100 μL wells of ELISA plates (Nunc). After drying, 100 μL of diluted serum (1:100) was added and incubated at room temperature. Plates were then washed and an HRP-labeled monoclonal antibody to human IgG4 (fc-specific, Acris antibodies CN AM20252HR-N) was added and reacted with the TMB substrate. The results were read by an ELISA reader EL-800 (Biotek Instruments) and expressed as mcg/mL. Normalization was obtained using graded concentrations of Human IgG4 Kappa (Millipore CN AG508).

Statistical analysis

Categorical variables were analyzed by Fisher's exact test. Kaplan-Meier survival curves were plotted to assess the temporal rate of events and the log rank test was computed for the comparison between survival free durations. Odds ratio and 95%CI were computed for all compared variables. The analysis was performed using MedCalc software (version 12.2.1.0, Mariakerke, Belgium). A two-tailed $P < 0.05$ was considered statistically significant.

RESULTS

Prevalence of the DN status using two ELISA assays

Out of 188 sera obtained from patients with LOR during regular IFX therapy, 67 were randomly selected for comparative analysis using the two techniques (anti-lambda and double antigen ELISA, Figure 1). In this analysis, 27/67 sera (40%) tested IFX-/ATI- with double antigen-ELISA compared to 6/67 (9%) with anti-lambda-ELISA ($P < 0.001$, Fisher's exact test). The calculated number needed to test (NNT) for a false-negative DN result by double-antigen ELISA was 3.2. As depicted in Figure 2, when applying anti-lambda-ELISA to the 27 sera that were IFX-/ATI- with the double antigen assay, only 6 (22%) remained DN, while 12 (44%) were actually IFX positive (IFX+ATI-), 8 (30%) were ATI positive (IFX-ATI+) and one serum (4%) was double positive (IFX+ATI+).

Double negativity on 1:10 dilution anti-lambda ELISA

When investigating the occurrence of double negativity regardless of patients' response status, we found that only 92 of the 1495 sera (6%) analyzed at our center between 2009-2013 were DN by anti-lambda ELISA (Figure 1). To examine whether some of these DN sera represented low-titer ATI or low level IFX, we randomly selected 45 DN sera and re-tested them at a 1:10 dilution to increase analytical sensitivity (compared with standard anti-lambda testing using 1:100 serum dilution). Upon this 1:10 dilution test, 24 (53%) DN sera became IFX positive (IFX+ATI-), 15 (33%) were double positive (IFX+ATI+) and 5 (11%) were ATI positive (IFX-ATI+). Only one serum (2%) retained its double negativity on 1:10 dilution (Figure 3). This transformation into detectable levels on 1:10 dilution was primarily caused by the fact that all 30 sera of healthy controls unexposed to IFX remained DN when tested by 1:10 dilution, but with lower detection cut-off levels.

Determination of IgG4 vs IgG1 ATI

IgG4 are monovalent antibodies (as opposed to the bivalent IgG1), and are thereby detectable by the anti-lambda ELISA, rather than by the double antigen assay[18]. Therefore, we assumed that a DN status on double antigen ELISA might be a result of non-detection of IgG4 ATI. To test this, we analyzed five sera that were DN by the double antigen ELISA and ATI positive by the anti-lambda ELISA (IFX-ATI-), as well as five sera that were ATI positive on both assays (IFX-ATI+). Contrary to our assumption, IgG4 levels were higher among the double antigen ELISA ATI+ positive sera (Median 6.6, IQR 0.9-7.4 *vs* median 0.5, IQR 0.07-0.97, $P = 0.047$, respectively).

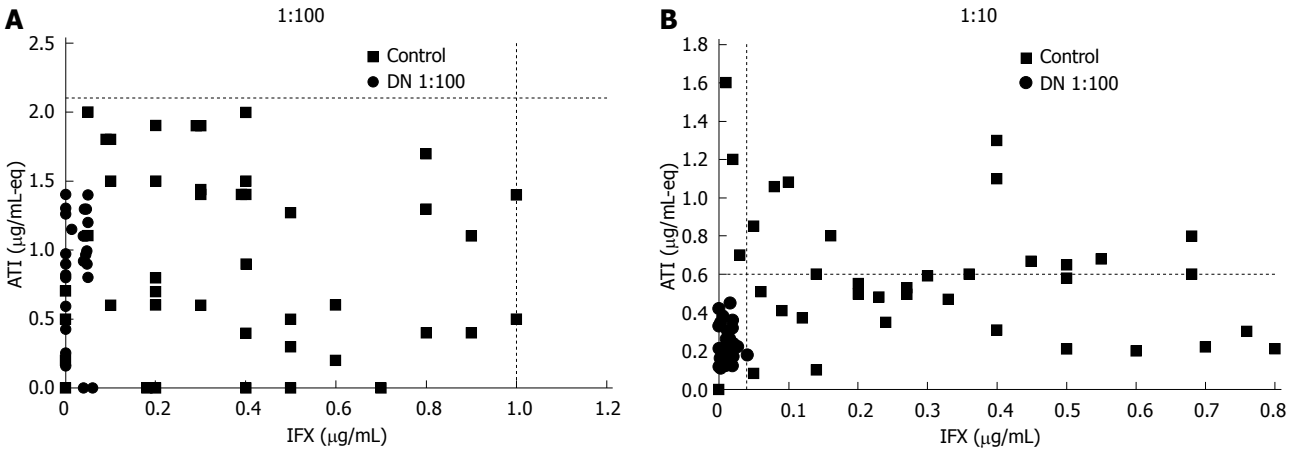

Figure 3 Re-testing of double negative sera to increase analytical sensitivity. To examine whether some of the double negative sera represented low-titer antibodies to infliximab or low-level infliximab, we randomly selected 45 double negative sera and re-tested them at a 1:10 dilution to increase analytical sensitivity (compared with standard anti-lambda testing using 1:100 serum dilution). A: IFX and ATI values of double negative *vs* healthy controls' sera, analyzed at 1:100 dilution anti-lambda ELISA. Cut off values for double negativity: IFX < 1 μg/mL, ATI < 2.1 μg/mL-eq. DN sera - black squares, healthy controls - circles; B: IFX and ATI values of sera DN on 1:100 dilution *vs* healthy controls analyzed at 1:10 dilution anti-lambda ELISA. Cut off values for double negativity: IFX < 0.04 μg/mL, ATI < 0.6 μg/mL-eq. Previously DN sera (on 1:100 dilution) - black squares, healthy controls - circles. DN: Double negative; IFX: Infliximab; ATI: Antibodies to infliximab.

Subsequent ATI formation and clinical response rate in DN patients

To investigate whether DN status is a harbinger of pending immunogenicity, we sought to determine whether patients with DN sera were predisposed to develop ATI compared with patients with measurable IFX (IFX+ATI-). During the study period, 44 out of 155 patients on standard IFX regiment had at least one DN serum sample determined by anti-lambda ELISA (Figure 1). Fourteen of them were excluded from analysis because of missing data (10 were inconsistently followed, two were lost to follow up after the DN event and two received infusions outside our center). Thus, 30 patients (25 CD, 5 UC) were included and matched with 30 controls (27 CD, 3 UC). Median follow up time was 21 ± 25.1 mo *vs* 20.75 ± 25.5 mo, respectively, $P = 0.97$. The patients' demographic and clinical characteristics are presented in Table 1.

ATI formation was significantly more frequent among the DN group compared with controls (OR = 11, 95%CI: 3.3-36.8, $P \leqslant 0.001$). In 9/30 (30%) DN patients who developed non-transient ATI, ATI formation occurred both before and after the event at which the DN serum was obtained (Figure 4), supporting the fact that a DN result may represent a particular time-point along the two curves of ATI titer rise and IFX drug level decline.

To investigate the temporal evolution of immunogenicity in DN patients, Kaplan-Meier analysis was performed. ATI free survival was significantly longer among controls (log rank test, $P < 0.001$, Figure 5A) than among DN cases. Of note, ATI appearance prior to the DN status was disregarded in this analysis. Nevertheless, when considering ATI existence before the starting point as positive, similar results were obtained (log rank test, $P < 0.001$). Secondary LOR was also more frequent among DN cases (OR = 4.66 95%CI: 1.57-13.86, $P = 0.006$) and survival free of secondary LOR was significantly shorter than among controls ($P = 0.02$, log rank test, Figure 5B).

DISCUSSION

A substantial portion of IFX treated patients develop low trough levels of IFX in the absence of measurable ATI (IFX-ATI-), *i.e.*, a DN status. Several studies have demonstrated that among patients with LOR, a DN result is more prevalent than antibody positive sera[10,19]. The actual mechanism of LOR remains unclear in most such cases, and the role - if any - of immunogenicity in instigating this phenomenon remains to be determined. In addition to assay limitations and irregular sampling time-points, DN status has been attributed to non-immune clearance of anti-TNF, high tissue inflammatory burden "absorbing" anti-TNF drug and temporal "window phenomenon", which refers to sampling when all drug-ATI complexes have been cleared[5,11,20].

Studies that employed double antigen ELISA assay reported 20%-40% of the patients as DN, regardless of response status[7,9,21,22]. Vande Casteele *et al*[12] recently demonstrated a prevalence of only 11% using the homogeneous mobility shift assay (HMSA). Little data exists comparing different methods for IFX and ATI level measurement. Steenholdt *et al*[23] recently demonstrated a lower detection rate of ATI using double-antigen ELISA than by other essays. In the current study, double negativity was significantly more prevalent when using the double antigen ELISA compared with the anti-lambda ELISA (40% *vs* 9%, $P < 0.001$). Furthermore, when applying anti-lambda ELISA to the sera that were IFX-/ATI- by the double antigen assay, only six (22%) remained DN.

Table 1 Background disposition and clinical characteristics *n* (%)

Parameter	Cases	Controls	*P* value	OR (95%CI)
Gender			0.19	2.0 (0.70-5.70)
Male	15 (50)	10 (33.3)		
Female	15 (50)	20 (66.6)		
Type of IBD			0.45	0.56 (0.12-2.57)
CD	25 (83.3)	27 (90.0)		
UC	5 (16.7)	3 (10.0)		
Duration of IFX therapy[1] (mo)	21 ± 25.1	20.75 ± 25.5	0.97	
Concomitant therapy	11 (36.7)	11 (36.7)	1.00	1.00 (0.35-2.86)
Episodic therapy	6 (20)	6 (20.0)	1.00	1.00 (0.28-3.54)
Median age (yr)	33 ± 15.2	28.5 ± 10.7	0.24	
Median disease duration (yr)	10.5 ± 9	9 ± 7.7	0.65	
Median age at diagnosis (yr)	22 ± 12.4	20 ± 9.7	0.19	
CD - disease location				
Ileal	9 (36)	8 (29.7)	0.62	1.30 (0.41-4.30)
Ileo-colonic	8 (32)	11 (40.7)	0.51	0.68 (0.22-2.10)
Colonic	8 (32)	8 (29.6)	0.85	1.10 (0.34-3.63)
CD - upper GI involvement	2 (8)	2 (7.4)	0.93	1.08 (0.14-8.40)
CD - anal/perianal involvement	13 (52)	14 (51.8)	0.99	1.00 (0.34-3.00)
CD - disease behavior				
Non stricturing non penetrating	12 (48)	12 (44.5)	0.79	1.15 (0.38-3.43)
Stricturing	8 (32)	10 (37.0)	0.70	0.80 (0.25-2.50)
Penetrating	5 (20)	5 (18.5)	0.89	1.10 (0.28-4.40)
UC - disease location				
Proctitis	0	0		
Left-sided colitis	2 (40)	1 (33.3)		
Extensive colitis	3 (60)	2 (66.7)		
Extra-intestinal manifestations	15 (50)	15 (50.0)	1.00	1.00 (0.36-2.75)
Smoking	2 (6.7)	5 (16.7)	0.24	0.30 (0.06-2.00)
Immunomodulator therapy prior to infliximab therapy	26 (86.7)	23 (76.7)	0.32	1.97 (0.51-7.63)
Adalimumab therapy prior to infliximab therapy	4 (13.3)	0	0.12	10.30 (0.53-201.00)
Surgery prior to infliximab therapy	8 (26.7)	7 (23.3)	0.76	1.19 (0.37-3.85)

[1]All patients received infliximab infusions at a standard protocol of 5 mg/kg at 0, 2, 6 and every 8 wk. IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis; GI: Gastro-intestinal.

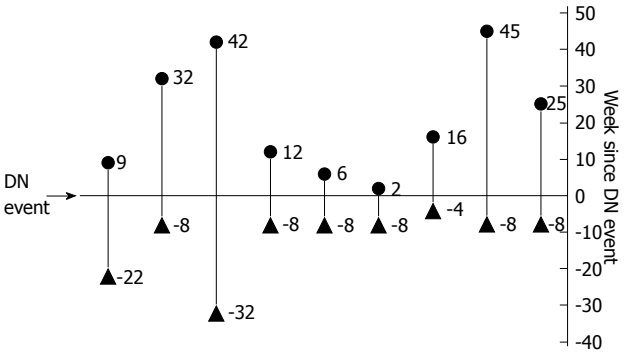

Figure 4 Temporal formation of antibodies to infliximab in nine patients in whom antibodies to infliximab positive sera preceded double negative sera. ATI formation before DN event (triangles), ATI formation after DN event (circles). ATI: Antibodies to infliximab; DN: Double negative.

We assumed that the higher frequency of double negativity using double antigen ELISA stems at least partly from false negative detection of IFX or ATI. As IgG4-ATI levels were not higher among the double antigen ELISA DN sera, double negativity cannot be attributed to the technical inability of double antigen ELISA to detect ATI in patients with a predominance of IgG4-ATI.

Interestingly, only one serum out of 45 examined with 1:100 dilution anti-lambda ELISA retained its double negativity at 1:10 dilution anti-lambda. The other sera became mostly IFX+ATI- or IFX-ATI+. The fact that almost all sera "lost" their DN status at 1:10 dilution implied that at least part of this phenomenon probably arises from low drug and ATI levels close to the detection threshold of the more sensitive anti-lambda assay. These sera may reflect a transitional state of immunological equilibrium between antibody-mediated IFX clearance and endogenous ATI production, rather than genuine non-immunological drug clearance. Notably, in clinical practice, physicians should be aware that some patients may present with DN status at trough merely because of arriving late for a delayed infusion. Such cases were excluded from the present work.

Few studies have addressed the question of subsequent ATI development in patients with DN sera. Hanauer *et al*[7] demonstrated that only 2.5% of DN patients turned ATI+ at week 76, although IFX infusions were halted at week 46. By contrast, Seow *et al*[9] showed that 77% of DN UC patients later developed ATI, regardless of response status. In our study, double negativity was also predictive of future non-transient ATI formation. This re-enforced our conclusion that DN status is an immunologically

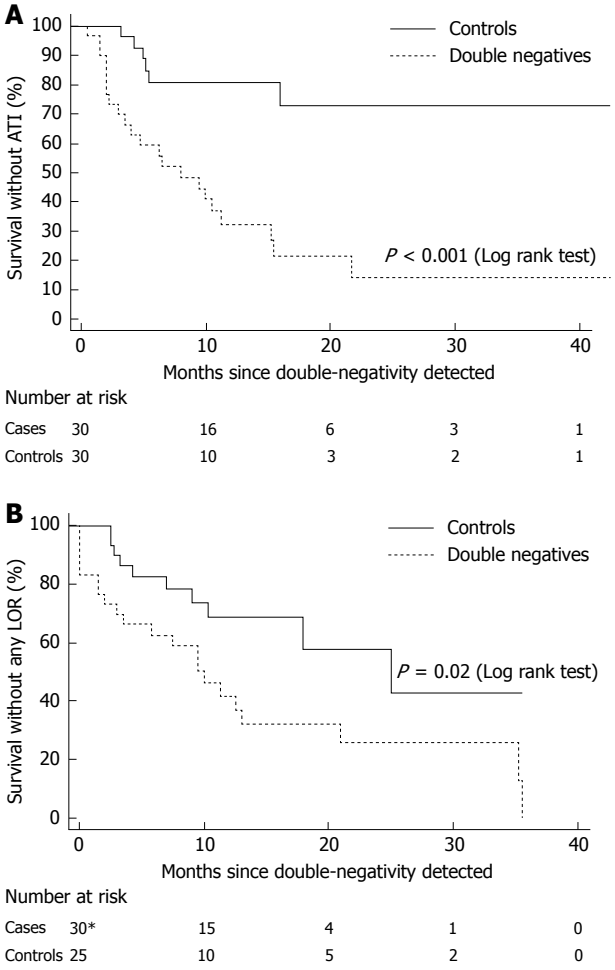

Figure 5 Survival free of antibodies to infliximab and loss of response. A: ATI development in cases *vs* controls; B: Secondary LOR in cases *vs* controls. The asterisk shows that five of the controls experienced secondary LOR at a time-point comparable to the double negative event. ATI: Antibodies to infliximab; LOR: Loss of response.

mediated phenomenon, albeit with low titer antibodies close or below the detection level of the assay when employed by certain specifications. As previously demonstrated, transient ATI had little clinical and immunological significance[8,12,13].

There are several limitations to our study. Primarily, the results were obtained with the double antigen ELISA and the anti-lambda ELISA; however, corroborating studies using other assays, such as HMSA, are pertinent. Secondly, because treating physicians were not blinded to the results, one cannot exclude that the DN status of the sera analyzed may have influenced clinical management. However, LOR was defined per clinical indexes and constituted only the secondary outcome. Finally, previous events of positive ATI might influence future ATI formation. To neutralize such past effects, we performed an analysis incorporating former ATI events as if they occurred at time zero, which yielded similar results.

In conclusion, the type of assay employed influences the occurrence of DN status. DN is rarely observed when LOR patients' sera are analyzed by the sensitive anti-lambda assay, and in many cases it probably results from low-level immunogenicity rather than elusive non-immunogenic mechanisms. Further studies are required to better assess the immunological processes leading to the absence of both drug and ATI, to investigate possible drug clearance pathways and to define appropriate interventions in these patients.

COMMENTS

Background

Antibodies to infliximab (ATI) correlate with lower infliximab (IFX) trough levels and loss of response (LOR). However, 10%-60% of LOR patients have low IFX levels in the absence of ATI, which are designated double negative (DN) status.

Research frontiers

The clinical and immunological significance of the DN status is currently unknown.

Innovations and breakthroughs

Only 9% of sera obtained at time of LOR were DN by anti-lambda ELISA compared with 40% with double antigen ELISA ($P < 0.001$, Fisher's Exact test). Re-testing with 1:10 dilution converted most of the DN results into IFX+ and /or ATI+ status. Patients with DN status had shorter survival free of non-transient ATI compared with matched controls (log rank test, $P < 0.001$).

Applications

The type of assay employed influences the occurrence of DN status. It is rarely observed when LOR patients' sera are analyzed by the sensitive anti-lambda assay, and in many cases it probably results from low-level immunogenicity rather than non-immunogenic mechanisms. Further studies are required to better assess the immunological processes leading to the absence of both drug and ATI, to investigate possible drug clearance pathways and to define appropriate interventions in these patients.

Terminology

DN status: A serum sample that is negative for both infliximab and ATI (IFX-ATI-). Double antigen ELISA: A commercially available ELISA assay for the detection of ATI, which incorporates infliximab as the detection antibody. Anti-lambda ELISA assay: An in-house developed ELISA assay for the detection of ATI, which incorporates an anti-human λ chain antibody as the detection antibody. LOR: Loss of clinical response to infliximab therapy.

Peer-review

This manuscript evaluates the prevalence and clinical significance of DN status among infliximab-treated inflammatory bowel disease patients. The article is well written and is suitable to be published.

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ORIGINAL ARTICLE

Prospective Study

Human cytomegalovirus and Epstein-Barr virus infection in inflammatory bowel disease: Need for mucosal viral load measurement

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Author contributions: Ciccocioppo R designed the study, was in charge of the diagnostic and therapeutic management of the patients, analyzed and interpreted the data, wrote the manuscript, and obtained funding. She has approved the final draft submitted; Racca F collected and analyzed the data. She has approved the final draft submitted; Paolucci S and Campanini G performed the quantitative real-time PCR analysis; They have approved the final draft submitted; Pozzi L carried out the endoscopic examinations with appropriate biopsy sampling. She has approved the final draft submitted; Betti E was involved in collecting patients' clinical data and technical support. She has approved the final draft submitted; Riboni R performed the immunohistochemistry analysis. She has approved the final draft submitted; Vanoli A analyzed the immunohistochemistry results. He has approved the final draft submitted; Baldanti F participated in the management of patients and in drafting the manuscript. He has approved the final draft submitted; Corazza GR was in charge of the management of the patients and critically revised the article for important intellectual content, he has approved the final draft submitted.
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Abstract

AIM: To evaluate the best diagnostic technique and risk factors of the human Cytomegalovirus (HCMV) and Epstein-Barr virus (EBV) infection in inflammatory bowel disease (IBD).

METHODS: A cohort of 40 IBD patients (17 refractory) and 40 controls underwent peripheral blood and endoscopic colonic mucosal sample harvest. Viral infection was assessed by quantitative real-time polymerase chain reaction and immunohistochemistry, and correlations with clinical and endoscopic indexes of activity, and risk factors were investigated.

RESULTS: All refractory patients carried detectable levels of HCMV and/or EBV mucosal load as compared

to 13/23 (56.5%) non-refractory and 13/40 (32.5%) controls. The median DNA value was significantly higher in refractory (HCMV 286 and EBV 5.440 copies/10^5 cells) than in non-refractory (HCMV 0 and EBV 6 copies/10^5 cells; $P < 0.05$ and < 0.001) IBD patients and controls (HCMV and EBV 0 copies/10^5 cells; $P < 0.001$ for both). Refractory patients showed DNA peak values $\geqslant 10^3$ copies/10^5 cells in diseased mucosa in comparison to non-diseased mucosa ($P < 0.0121$ for HCMV and < 0.0004 for EBV), while non-refractory patients and controls invariably displayed levels below this threshold, thus allowing us to differentiate viral colitis from mucosal infection. Moreover, the mucosal load positively correlated with the values found in the peripheral blood, whilst no correlation with the number of positive cells at immunohistochemistry was found. Steroid use was identified as a significant risk factor for both HCMV ($P = 0.018$) and EBV ($P = 0.002$) colitis. Finally, a course of specific antiviral therapy with ganciclovir was successful in all refractory patients with HCMV colitis, whilst refractory patients with EBV colitis did not show any improvement despite steroid tapering and discontinuation of the other medications.

CONCLUSION: Viral colitis appeared to contribute to mucosal lesions in refractory IBD, and its correct diagnosis and management require quantitative real-time polymerase chain reaction assay of mucosal specimens.

Key words: Inflammatory bowel disease; Quantitative real-time polymerase chain reaction; Steroid therapy; Refractory; Viral infection

Core tip: This study investigated the presence of human Cytomegalovirus and Epstein-Barr virus (EBV) infection in patients with refractory and non-refractory inflammatory bowel disease (IBD). We identified quantitative real-time polymerase chain reaction assay of mucosal specimens as the best diagnostic technique. This allowed us to distinguish between viral colitis and infection through the identification of a cutoff value. All refractory IBD patients carried the highest mucosal viral loads, which correlated with the severity of mucosal damage and endoscopic activity. EBV infection was the most prevalent. Finally, steroid therapy was identified as a significant risk factor for viral colitis.

Ciccocioppo R, Racca F, Paolucci S, Campanini G, Pozzi L, Betti E, Riboni R, Vanoli A, Baldanti F, Corazza GR. Human cytomegalovirus and Epstein-Barr virus infection in inflammatory bowel disease: Need for mucosal viral load measurement. *World J Gastroenterol* 2015; 21(6): 1915-1926 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i6/1915.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i6.1915

INTRODUCTION

Growing evidence highlights the role of early treatment of inflammatory bowel disease (IBD) by means of a more aggressive therapy, in order to achieve mucosal healing and prevent disease progression[1]. As a consequence, a sizeable number of patients are on immunosuppressive and/or biological drugs which, in turn, lead to an increased risk of opportunistic infections[2,3]. Among these, the widespread human cytomegalovirus (HCMV) and Epstein-Barr virus (EBV) are capable of establishing latency in target cells and reactivating in cases of reduced host immunity[4], giving rise to both systemic and end-organ disease, which can also be localized localized to the gastrointestinal tract[5,6]. In addition, an increased risk of lymphoma has been recently found in IBD patients under immunosuppressive and/or immunomodulator therapies, especially among young males under thiopurines, where a role for primary EBV infection has been proposed[7], as in the case of the post-transplant lymphoproliferative disease[8]. A further condition now possibly associated with both HCMV and EBV infections is hemophagocytic lymphohistiocytosis[9].

To date, the information available on the frequency, role and risk factors of HCMV and EBV in IBD exacerbation and their diagnostic and therapeutic approaches is conflicting[10,11]. The causes for this discrepancy lie in the differences amongst the patients enrolled, the diagnostic methods applied, and the retrospective design of the majority of studies. Herein, we prospectively evaluated the presence of HCMV and EBV infections in a cohort of IBD patients in comparison to control subjects, through both immunohistochemistry and quantitative real-time polymerase chain reaction (PCR), in order to investigate the contribution of these opportunistic viral infections to disease activity and the risk factors for their reactivation.

MATERIALS AND METHODS

Study population

From September 2011 to February 2013, patients suffering from Crohn's disease (CD) and ulcerative colitis (UC), both responders and non-responders to conventional therapies, and diagnosed on the basis of widely accepted criteria[12,13], were prospectively enrolled. For the non-responder group, steroid-refractoriness was defined as the persistence of active disease despite prednisolone, or equivalent, of up to 0.75 mg/kg per day over a period of four weeks[12,13]; primary and secondary non-response to anti-tumor necrosis factor-α agents, *i.e.*, infliximab and adalimumab, was considered as the lack of clinical improvement with induction therapy or re-

currence of disease activity during maintenance therapy despite an appropriate interval adjustment and dose escalation with exclusion of concomitant conditions, respectively[14]; and resistance to immunosuppressive therapy, *i.e.*, Azathioprine, after the dosage adjustment was carried out on the basis of the erythrocyte levels of the active metabolite, 6-thioguanine nucleotide[15]. Finally, for those patients under combination therapies, refractoriness was defined as the persistence of active disease despite a treatment duration of at least four weeks. All patients underwent lower endoscopy as part of their diagnostic workup for disease relapse or follow-up. Patient assessment was performed according to the Montreal classification[16], and also included: clinical examination, body mass index calculation, evaluation of smoking habits, routine laboratory tests, and assessment of both clinical (CD activity index: CDAI[17] and colitis activity index: CAI[18]) and endoscopic (SES-CD[19] and Baron[20]) indexes of activity. As a control group, sex- and age-matched subjects undergoing lower endoscopy for irritable bowel syndrome or screening for polyps, who were not taking any drugs, were recruited. The presence of concomitant autoimmune diseases, primary immunodeficiencies, cancer or organ failure was considered exclusion criteria.

Quantitation of HCMV and EBV DNA

For each patient and control, the HCMV and EBV load was assessed in terms of DNA copies on both freshly collected peripheral blood samples and endoscopic specimens harvested from all colonic segments (right, transverse, descending, and sigma-rectum), by quantitative real-time PCR technique as previously reported[21]. Specifically, in the IBD group, biopsies were taken from both inflamed and healthy mucosa as assessed during the endoscopic examination, that is from the edge of the ulcers and the nearby damaged zones for the former and at least 20 cm away from the affected areas for the latter. Viral DNA extraction was performed by using the NucliSENS® easyMAG® kit (BioMérieux; Lyon, France). Results were expressed as viral DNA copies/ml blood and copies/10^5 cells. Normalization of HCMV and EBV DNA load in tissue samples was obtained by quantitative determination of β_2-microglobulin gene[22]. The lower detection limit was 10 DNA input target DNA copies[21,22].

Immunohistochemistry

Mucosal specimens harvested in parallel from the same areas as those for the PCR assay, were fixed in 10% neutral buffered formalin and paraffine-embedded. Sections (5 μm) were transferred to pre-treated glass slides (DAKO, Denmark) and stored at 37 ℃ overnight. The hematoxylin-eosin staining was performed following standard protocol, while the specific immunostaining for HCMV and EBV was carried out on seriate sections after microwave demasking treatment. The slides were then washed and incubated for two hours with the following mouse monoclonal antibodies: anti-HCMV (clones CCH2 and DDG9 that recognize HCMV immediate-early and early antigens, respectively, at 1:300 dilution; DAKO), and anti-LMP1 (clone CS1-4 reactive to EBV latent membrane protein-1, at 1:100 dilution; DAKO). Finally, a universal biotinylated secondary antibody (DAKO) was applied, followed by the usual reactions to allow color development (liquid DAB + Substrate Chromogen System; DAKO) and counterstaining with Harris' hematoxylin. Appropriate positive controls and non-immune protein-negative controls were used, and positive cells were evaluated by a pathologist blinded to patient diagnosis and clinical disease status.

Statistical analysis

Baseline demographic and disease features are presented by using descriptive statistics. As such, continuous variables were described as median, while categorical variables were expressed as counts and percentages, all with range. The univariate analysis was carried out to compare data between groups by applying the following tests: Fisher's exact test or Wilcoxon matched pairs signed-ranks, and Mann-Whitney or Kruskal-Wallis, for categorical and continuous variables, respectively, as appropriate. The multiple regression analysis was performed to assess the association between possible risk factors and the occurrence of viral infections. The individual demographic and clinical variables (including gender, age, body mass index, smoking habits, duration of both therapies and disease, concomitant immunosuppressive therapy, clinical and endoscopic indexes of activity) were considered as risk factors. The Spearman rank correlation test was applied to measure the association between continuous variables, and the calculation of the OR at 95%CI was assessed. GraphPad InStat 3.0 was used for computation. A 2-sided *P*-value $\leqslant$ 0.05 was considered statistically significant.

Ethical considerations

All samples were collected for diagnostic purposes and only residual aliquots of both peripheral blood and mucosal specimens from control subjects were used, after the subjects had signed the informed consent, and in accordance with the recommendations of the local Bio-Ethics Committee.

RESULTS

Study population

A total of 17 refractory and 23 non-refractory IBD patients, and 40 control subjects whose clinical features are presented in Table 1 were consecutively recruited at the Department of Internal Medicine, IRCCS Policlinico San Matteo Foundation (Pavia, Italy). Specifically, all refractory patients were admitted to the hospital for severe active disease, while in the

Table 1 Demographic and clinical features of inflammatory bowel disease patients

	Responders	Refractory	Controls	*P* value
Number of patients	23	17	40	
Male/female	14/9	9/8	24/16	
Body mass index[1]	22.8 (19.9-26.4)	20.3 (16.4-22.5)	23.4 (18.9-27.3)	
Age (yr)[1]	45 (16-68)	50 (16-61)	46 (17-68)	NS
CDAI score[1]	145 (124-205)	383 (265-595)	NA	0.002
Number of patients	12	4		
Location	L1 = 2	L1 = 0		NS
	L2 = 3	L2 = 0		
	L3 = 7	L3 = 4		
	L4 = 0	L4 = 0		
CAI score[1]	6 (3.5-9.5)	10 (8.25-11)	NA	0.040
Number of patients	11	13		
Location	E1 = 2	E1 = 0		NS
	E2 = 3	E2 = 5		
	E3 = 6	E3 = 8		
Endoscopic activity indexes			NA	
SES-CD	6.2	10.6		0.010
Baron	1.4	2.6		0.050
Illness duration (yr)[1]	6.5 (1.75-11)	5 (1-7)	NA	NS
Systemic steroids	4	14	NA	0.001
Topical steroids	5	8	NA	NS
Biological agents	5	8	NA	NS
Azathioprine	7	5	NA	NS
Biological + steroid	0	6	NA	0.0032
Biological + azathioprine	1	4	NA	NS
Azathioprine + steroid	1	0	NA	NS

[1]The values are given as median (range). L1: Terminal ileum; L2: Colon; L3: Ileo-colon; L4: Upper gastrointestinal location; E1: Ulcerative proctitis; E2: Left sided UC; E3: Extensive UC; NA: Not-applicable; NS: Not significant; CDAI: Crohn's disease activity index. Systemic steroids included: Metilprednisolone and prednisone; topical steroids included: Budesonide and beclometasone dipropionate; biological agents included: Infliximab and adalimumab.

Table 2 Mucosal viral loads

	HCMV	EBV
Number of patients	15/80	32/80
Refractory	286	5.440
	(0-221.697)	(0-966.333)
Diseased mucosa	30.763	8.294
	(11.911-221.697)	(1.020-966.333)
	0	281
Non-diseased mucosa	(0-3)	(12-400)
Responders	0	6
	(0-273)	(0-973)
Diseased mucosa	0	9
	(0-273)	(0-973)
Non-diseased mucosa	0	4
	(0-2)	(0-63)
Controls	0	0
	(0-41)	(0-34)

The values are given as copies of DNA/10^5 cells and showed as median (range). HCMV: Human Cytomegalovirus; EBV: Epstein-Barr virus.

non-refractory group, the hospitalization rate was 39%.

Mucosal viral load

Detectable copies of viral DNA were found in all (17/17, 100%) refractory, 13 out of 23 (56.5%) non-refractory IBD patients, and 13 out of 40 (32.5%) controls, with HCMV DNA evident in four refractory and five non-refractory IBD patients, and two controls, EBV DNA found in nine refractory and eight non-refractory IBD patients, and 11 controls, and double positivity found only in four refractory patients. The median values with ranges of DNA copies of both viruses, as obtained by pooling together the data from all colonic segments of each patient and then, in the IBD groups, from both inflamed and non-inflamed mucosa, are shown in Table 2. It is worth of noting that the median values for both HCMV (Figure 1, panel A) and EBV (Figure 1, panel B) were significantly higher in refractory IBD compared to non-refractory IBD patients and controls, while non-refractory IBD patients did not show significantly different values compared to controls (Figure 1, panels A and B). Moreover, refractory IBD patients invariably showed DNA peak values $\geqslant 10^3$ copies/10^5 cells in at least one colonic segment, while patients with non-refractory IBD and controls displayed viral DNA peak levels below 10^3 and 10^2 copies/10^5 cells, respectively (Figure 1, panels A and B). Finally, upon analyzing the viral DNA loads within the refractory IBD group, the median values were found to be significantly higher in diseased *vs* non-diseased mucosa (Figure 1, panels C and D), whilst no difference was found when the median viral DNA levels of non-diseased mucosa were compared with those found in both non-refractory IBD patients and controls. It is worth noting that there was a positive correlation (r = 0.71 and 0.79 for HCMV and EBV, respectively) between the mucosal viral load in the refractory IBD and the degree of endoscopic activity (Table 1). In this regard, a narrow overlap between the extension and severity of mucosal le-

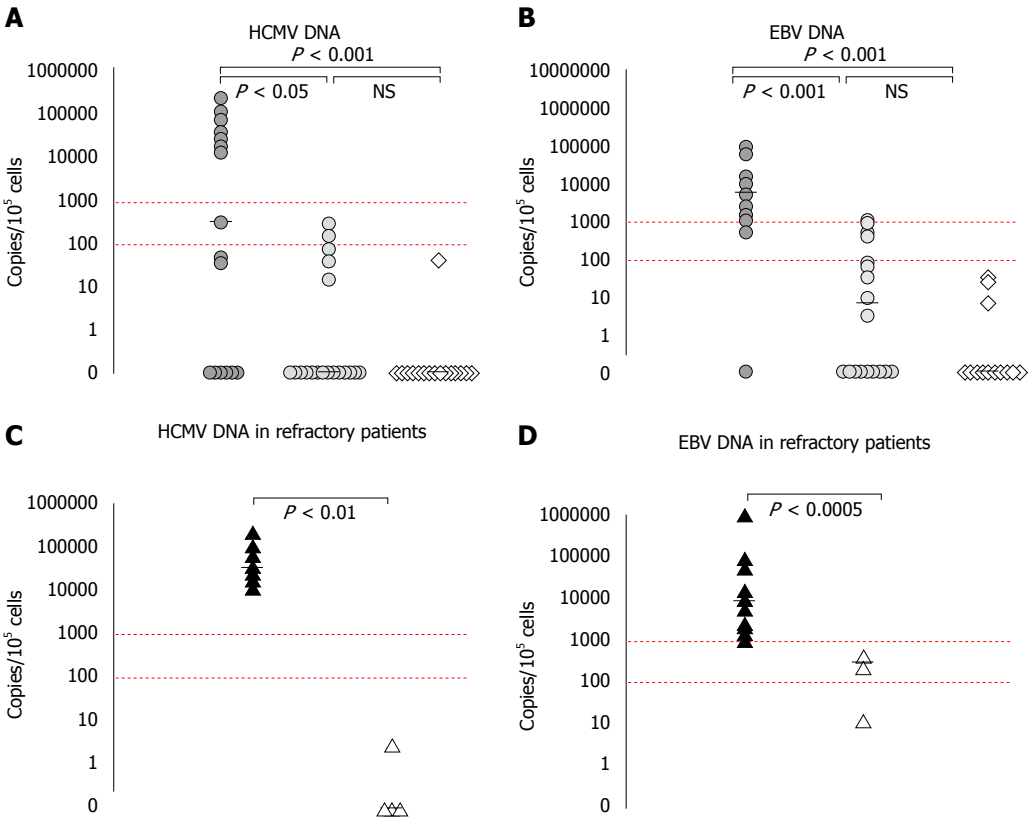

Figure 1 Refractory inflammatory bowel disease patients (full circles) showed statistically significant higher values in comparison with non-refractory patients (empty circles) and controls (empty rhombuses; panels A and B). A peculiar distribution is observed with non-refractory patients displaying values always below 10^3 and controls below 10^2 copies/10^5 cells (red dotted lines). Within the refractory group, the macroscopically diseased areas (full triangles) carried DNA viral loads invariably over 10^3 copies/10^5 cells compared to non-diseased mucosa (empty triangles; panels C and D). The black bars indicate the median values. NS: Not significant; HCMV: Human Cytomegalovirus; EBV: Epstein-Barr virus.

sions with the viral load distribution was invariably found (representative cases are shown in Figure 2). By contrast, no correlation between viral load and clinical indexes of disease activity and no preferential association between HCMV or EBV with UC or CD were observed.

Blood viral load

Viral DNA was detected in 11/17 patients with refractory IBD (median values for HCMV and EBV: 0 copies/mL, range: 300-26.000, and 100-7.900, respectively), 1/23 of non-refractory IBD (450 copies/mL), and none of the controls. Specifically, two refractory patients showed HCMV DNA, five refractory patients and one non-refractory patient had EBV DNA, and four refractory patients carried both DNAs. All these patients carried the same virus(es) at mucosal level, and a significant positive correlation between the values in the two compartments (r = 0.67 for HCMV and 0.61 for EBV) was observed. The sensitivity of the PCR assay performed on peripheral blood samples as compared to that on tissue samples, therefore, was 23% for HCMV and 45% for EBV, with a specificity of 100% for both, while the positive predictive value was 76.4% and 17.6%, and the negative predictive value was 65.0% and 80.9% for HCMV and EBV, respectively.

Histological and endoscopic features

As far as immunohistochemistry is concerned (representative cases shown in Figure 3), although the specimens were harvested from the same mucosal areas as those taken for the PCR assay, HCMV positive cells were found in only 11 cases (five had a DNA load > 10^3 copies/10^5 cells, and six were negative), while EBV positive cells were detected in 17 cases (five had a viral DNA load > 10^3 copies, eight < 10^3 copies/10^5 cells, and four negative), thus showing a sensitivity of 33% for both viruses, a specificity of 90% for HCMV and 0% for EBV, with positive predictive values of 66% and 80%, and negative predictive values of 71% and 0% for HCMV and EBV, respectively. No correlations between the mean number of positive cells and the level of mucosal viral load for either virus or the degree of endoscopic activity were found.

As regards the endoscopic features of refractory IBD patients, the mucosal lesions appeared indistinguishable from those of the underlying disease, despite the invariable presence of the superimposed viral end-organ disease, as evident in Figures 4, 5, and 6 showing representative cases. It is worth noting that mucosal healing was observed in all cases with isolated HCMV colitis (Figure 4, panel C and F) following specific antiviral therapy (see below). Conversely, no amelioration was evident in any of the cases carrying

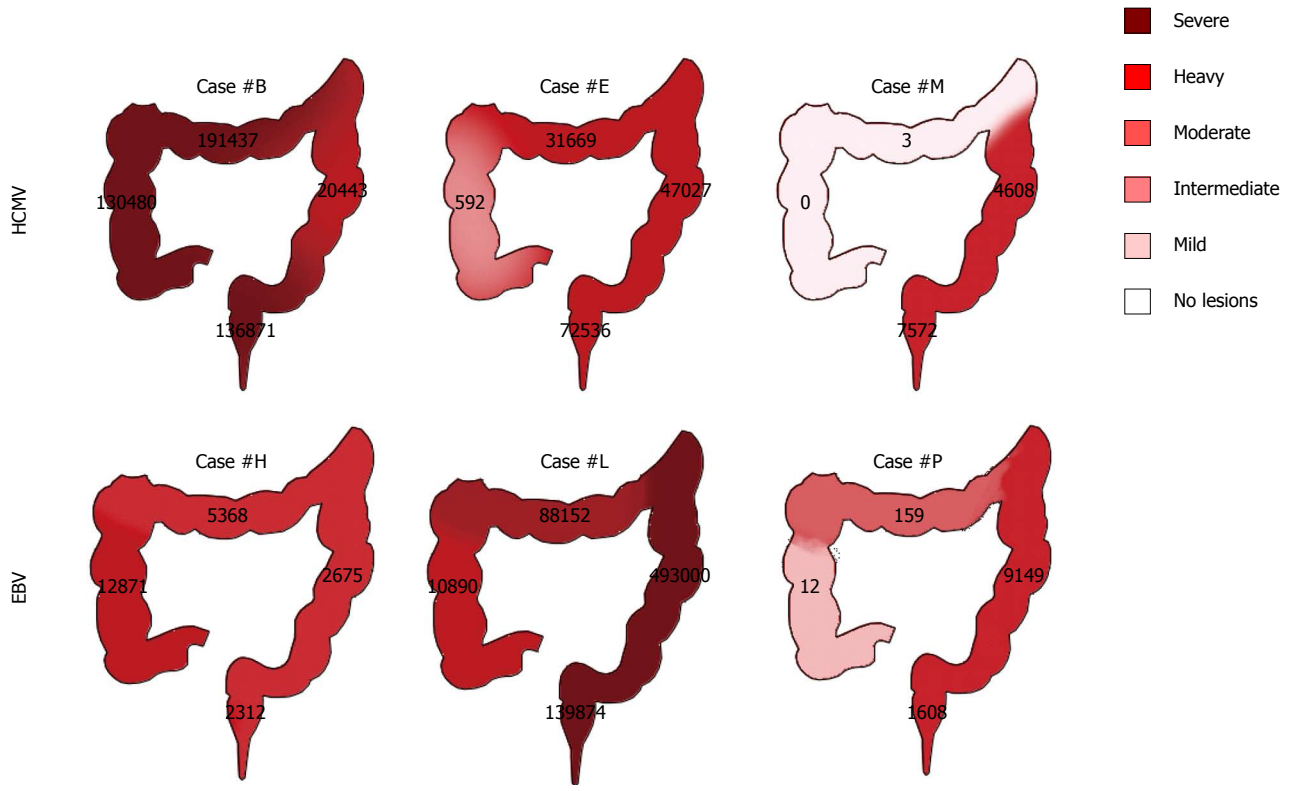

Figure 2 Six representative refractory patients with Human Cytomegalovirus - upper panels - and Epstein-Barr virus - lower panels - superimposed colitis. The distribution of the DNA viral loads (given as number of copies/10^5 cells) along the colon perfectly matches the distribution and severity of mucosal lesions as shown by the arbitrary red scale. HCMV: Human Cytomegalovirus; EBV: Epstein-Barr virus.

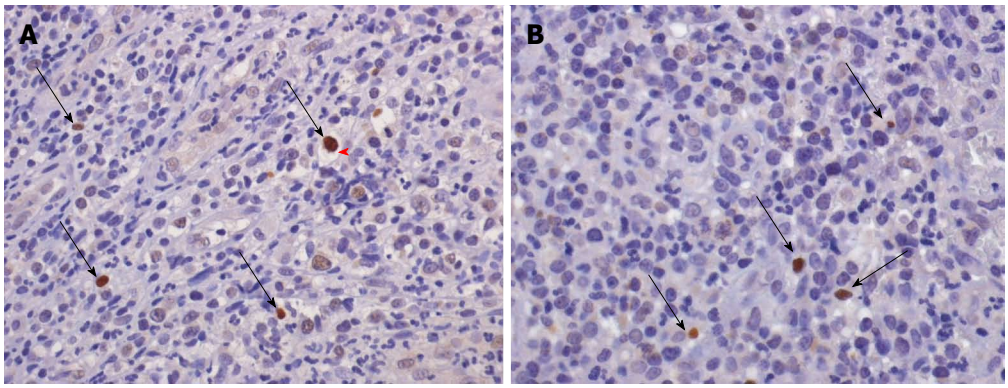

Figure 3 Pathognomonic "cytomegalic" cell (*i.e.*, enlarged cell surrounded by a light-coloured halo, red arrow) with a brown reactive nuclear inclusion (thin black arrows) and a few scattered positive cells for the human Cytomegalovirus are shown (panel A, immunoperoxidase-hematoxylin, original magnification × 250). Some positive cells with brown nuclei (thin black arrows) are evident following the specific staining for the Epstein-Barr virus nuclear antigen-1 (panel B, immunoperoxidase-hematoxylin, original magnification × 400).

both viruses (Figure 6, panels C and F), nor in most of those with EBV (Figure 5, panels E and F), except the patient (Figure 5, panel D) who underwent a course of treatment with rituximab (see below).

Impact of current therapies

Systemic steroid use was identified as a significant risk factor for both HCMV and EBV colitis (P = 0.018 and 0.0020, OR = 11.4 and 12, 95%CI: 1.23-106.11 and 2.37-60.67, respectively), while the use of biologic agents and topical steroids was closely related to EBV colitis (P = 0.021 and 0.008, OR = 7.8 and 10.2, 95%CI: 1.32-46.64 and 1.73-60.92, respectively). No significant correlation was found between an increased risk of development of viral end-organ disease and the use of immunosuppressants, the duration of both therapies and underlying disease, age, gender, smoking habits, or body mass index.

Patients' outcome

All refractory patients with HCMV colitis underwent a course of specific antiviral therapy with ganciclovir

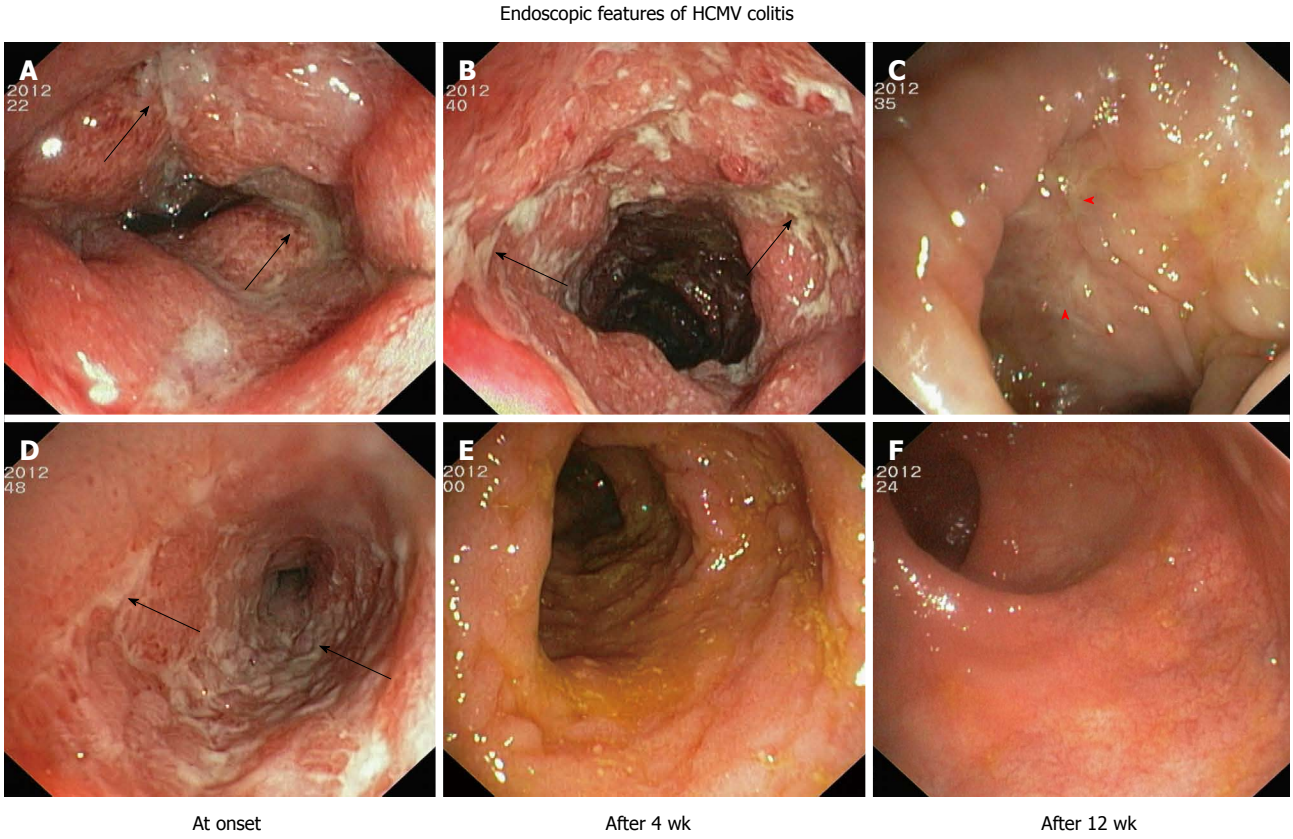

Figure 4 Presence of profound, round and longitudinal ulcers covered by a fibrino-purulent exudate (black arrows) and embedded in edematous and erythematous mucosa are clearly evident (panels A and D). The healing process, usually observed after three months from the end of specific antiviral therapy, may result in both white scars (red arrows, panel C) or restitution ad integrum (panel F), passing through a phase of slight improvement (panels B and E).

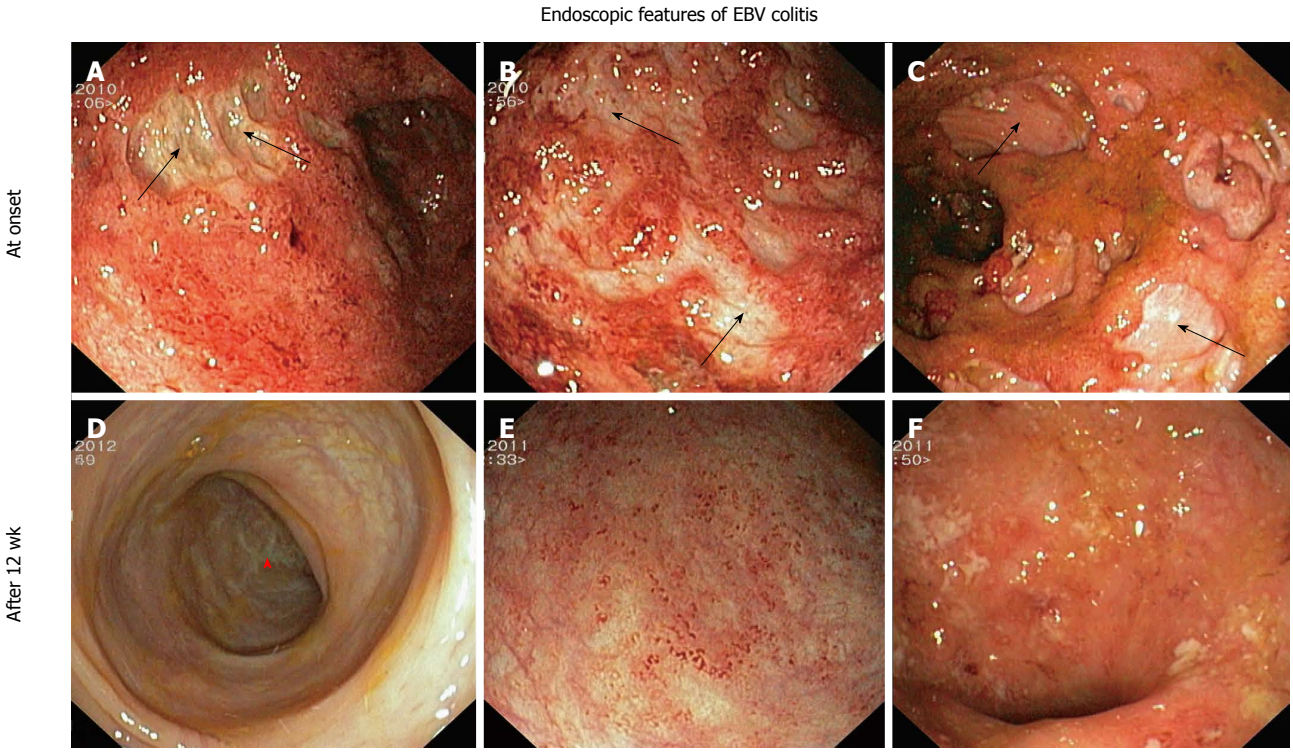

Figure 5 Presence of holes in the mucosa with exposure of the underlying muscular layer (black arrows), surrounded by granular and spontaneously bleeding zones, is clearly evident (panels A to C). After a three-month washout period from any therapy for the primary disease, the healing process with white scars (red arrow) was observed in the only patient who underwent a cycle with rituximab (panel D), whilst a slight or no improvement was observed in the other two representative cases.

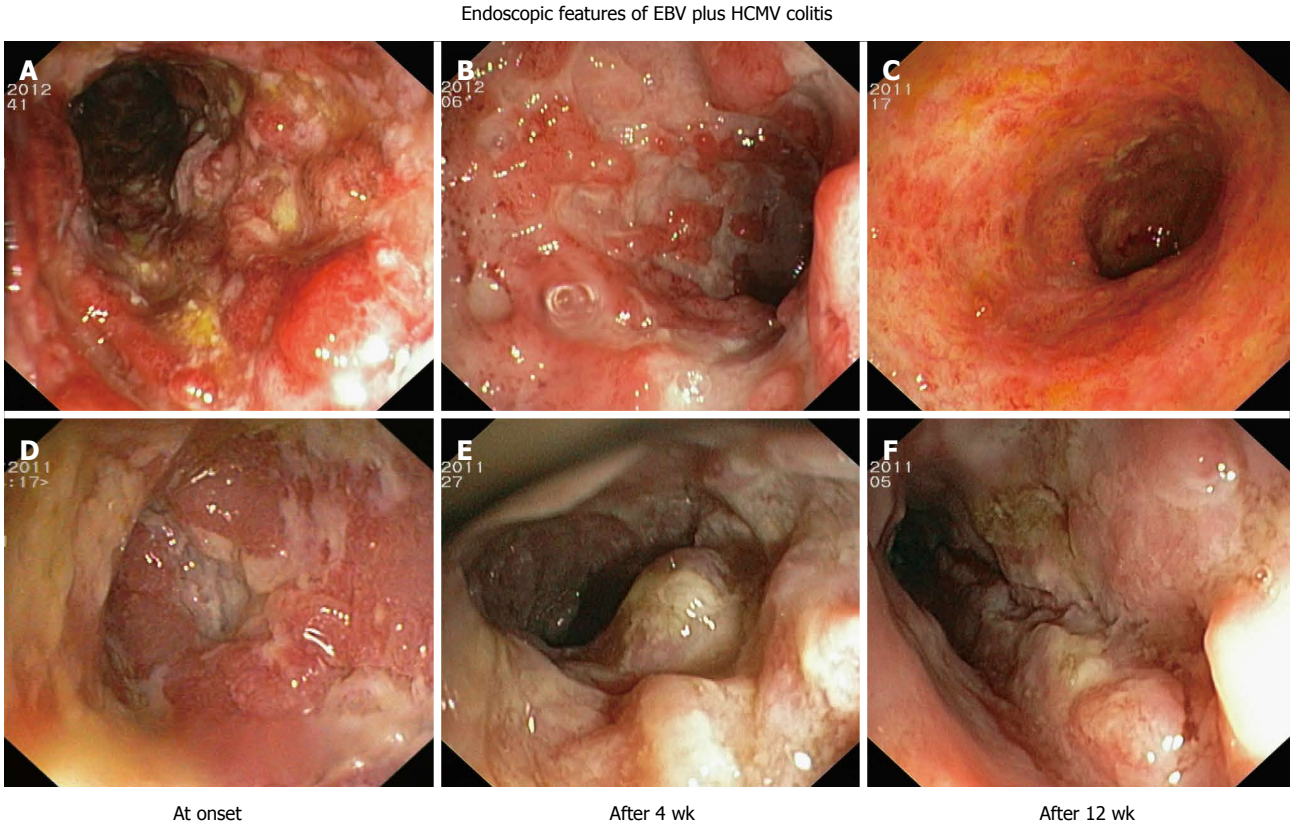

Figure 6 Severe lesions characterized by a pronounced, nodular-cobblestone appearance, punctuated by multiple, deep ulcerations (panels A, B, D, E), which did not heal (panels C, F), were found in those refractory patients with high DNA loads of both viruses.

(5 mg/kg bid iv) for three weeks[11], with monitoring of mucosal and blood viral load at the end of therapy and, following discharge, after four and 12 wk, as arbitrarily scheduled. All patients reached a satisfactory general condition with a sharp decrease in both clinical and endoscopic indexes of activity [median values: 249 (range: 166-302) and 10.37 (8.7-12.2) after four weeks, and 173 (132-209) and 7.2 (5.9-9.3) after 12 wk for CD patients, and 7 (range: 4-9) and 1 (2-1) after four weeks, and 5 (4-7) and 1 (1-2) after 12 wk for UC patients]. The viral mucosal DNA levels also decreased significantly (P = 0.031) by the end of therapy (Figure 7A), and remained stable afterwards (data not shown), with patients experiencing a good quality of life during a median follow-up period of 11 mo (range: 6-22). In contrast, refractory patients with EBV colitis did not show any improvement in either clinical or endoscopic activity indexes [median values: 396 (range: 281-595) and 11.49 (range: 8.9-12.4) after four weeks, and 295 (range: 351-244), and 10.44 (range: 9.0-11.7) after 12 wk for CD patients, and 11 (range: 8-13) and 2 (range: 2-3) after four weeks, and 11 (range: 8-12) and 2 (range: 2-3) after 12 wk for UC patients]. Likewise, no significant decrease of mucosal (or blood when positive) EBV DNA levels was observed, despite steroid tapering and discontinuation of the other medications in 9/15 patients (Figure 7B). Remarkably, in the only case where the patient underwent treatment with the anti-CD20 monoclonal antibody rituximab (375 mg/m^2 body surface iv weekly for 4 wk[8]), EBV DNA was cleared from both blood and colonic mucosa. However, after six months, a worsening of the patient's clinical condition characterized by fever and intestinal bleeding was observed, with mucosal EBV load > 10^3 copies/10^5 cells, resulting in a colectomy. In this regard, 7/9 refractory patients with EBV colitis underwent colectomy within a median period of 5 mo (range: 2-9), as well as 3/4 patients carrying both virus DNAs within a median period of 8 mo (range: 5-14), despite the fact that HCMV had been cleared by antiviral therapy. The remaining patients developed chronically active colitis, thus adversely affecting their quality of life. During the same follow-up period, only two non-refractory CD patients underwent intestinal resection due to strictures.

DISCUSSION

Despite a growing interest in opportunistic viral infections in IBD, several crucial points still remain unsolved, such as their prevalence and role in tissue damage during exacerbation, the best diagnostic and therapeutic approaches, as well as the risk factors[2,3,10,11]. The reported frequency of HCMV infection, for instance, ranged from 10%[23,24] to 36%[25,26], whilst that of EBV was higher, ranging from 41%[27]

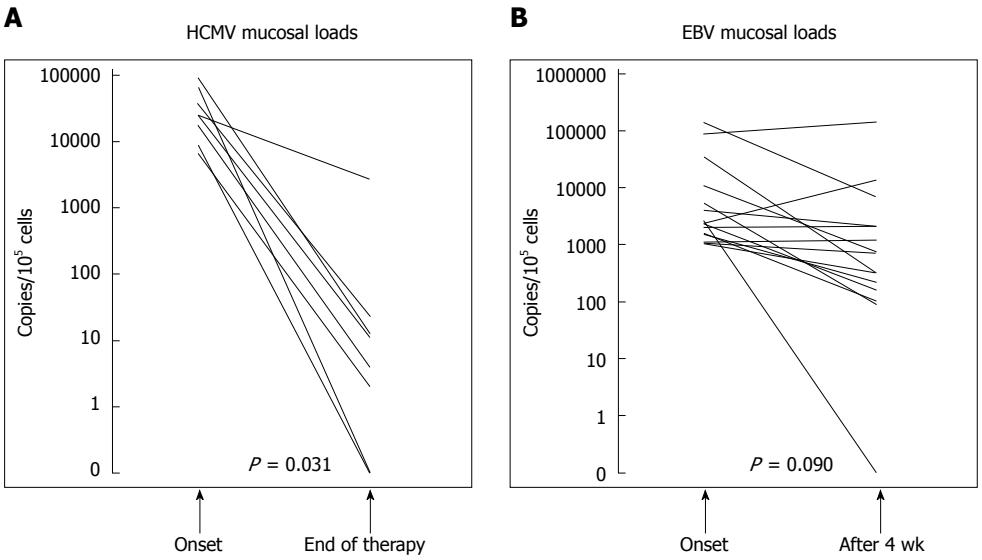

Figure 7 Median values of human Cytomegalovirus (panel A) and Epstein Barr virus (panel B) DNA levels of each refractory patient at onset and at the end of antiviral therapy in human Cytomegalovirus cases, and at four weeks after washout or reduction of current treatment in patients with Epstein-Barr virus infection. A dramatic decrease of mucosal viral loads was observed in all patients with HCMV colitis, except one who continued steroid therapy while taking ganciclovir. By contrast, no modification of mucosal viral load values was observed in any patient with EBV colitis, except the one who underwent a cycle of therapy with rituximab. HCMV: Human Cytomegalovirus; EBV: Epstein Barr virus.

to 64%[28]. In addition, most of the studies were retrospective, included surgery specimens, applied different techniques, and therefore, the results are not comparable. As far as the detection method is concerned, immunohistochemistry was the most widely used method[23,28-30], and it is given as the screening test in the decisional algorithm for the management of HCMV infection in IBD[31]. However, quantitative real-time PCR assay carried out on nucleic acids extracted from formalin-fixed paraffin-embedded intestinal specimens has recently proved to increase the sensitivity of immunohistochemistry[32]. Moreover, the application of this technique on fresh biological samples has emerged as the best technique[24,33-35] as it has the advantage of being highly sensitive, rapid, and reproducible. Our results fit in with this evidence, since when comparing the two techniques, immunohistochemistry showed low predictable values, both positive and negative, possibly due to the suboptimal specificity and sensitivity of the primary antibodies used and the lack of correlation with the lytic phase of the viruses. Similar results were obtained in congenital HCMV infection when the same primary antibodies were used[36], which hamper its usefulness in the management of IBD patients. By contrast, quantitative real-time PCR on freshly collected mucosal biopsies displayed not only a better performance, but also allowed us to distinguish between viral infection and colitis, since a positive result did not necessarily imply that the patient was suffering from an active HCMV or EBV end-organ disease. Accordingly, two main groups of mucosal viral load may be identified: that with peak values greater than 10^3 copies/10^5 cells, and that with values below this threshold. Since all refractory patients carried mucosal viral loads invariably greater than 10^3 copies/10^5 cells in at least in one colonic segment, it is conceivable that this indicates a symptomatic viral-related colitis that is superimposed on the underlying primary illness. Interestingly, our systematic sampling of all colonic segments made it possible to build up a map of the mucosal viral loads that perfectly overlaps with the severity of macroscopic lesions and correlates with the endoscopic indexes of activity. On the other hand, the presence of a number of DNA copies lower than 10^3/10^5 cells only in patients with quiescent disease might be related to the periodic viral reactivation that usually occurs even in healthy people without this triggers of symptoms[4]. However, on comparing the values found in non-refractory IBD patients with those found in control patients, a median mucosal peak value of 1 logarithm unit higher was observed in IBD patients. This finding could prove to be of some clinical use as a warning for closer follow-up and caution in prescribing those therapies that favor the disruption of the delicate balance between the host immune response and viral activity, which leads to precipitation or exacerbation of mucosal injury. A similar distinction between viral colitis and infection could not be achieved by previous studies even when applying PCR-based techniques, since the differences in expressing the results (as copies by microgram of total DNA extracted[24,34] or milligram of tissue[33]), the sensitivity of the test, and the lack of control population made it impossible to draw definite conclusions. In this regard, the possibility of measuring the DNA viral load in stool samples, thus avoiding an invasive endoscopic exam, has already been explored[37]. However, the lack of control populations, the limited number of cases enrolled and,

in most of all, the absence of an exact correspondence between the levels found in the mucosa and stool samples, did not allow the authors in question to differentiate between viral end-organ disease and reactivation/infection. Moreover, the presence of some technical limitations such as the need for a fresh, liquid stool sample made this diagnostic test unsuitable for the management of IBD patients. Finally, the search for circulating class M specific anti-virus antibodies is rather ineffective in detecting an active disease, since elevated levels can persist for up to two years after infection, and immunocompromised patients may not mount an IgM response[38].

We believe, therefore, that the previous definitions of HCMV or EBV infection and colitis, often used interchangeably, and simply referring to the evidence of viral positivity in colonic tissue of symptomatic patients by means of any technique[11,38], are confusing and should be assessed by applying the quantitative real-time PCR method on freshly harvested mucosal biopsies. However, further studies are warranted in order to optimize the mucosal sampling and assess the risk stratification in order to achieve early recognition and appropriate management of these conditions. It is worth noting that we confirmed[39,40] EBV as the most prevalent infection, since half (49%) of the IBD patients and almost all of the refractory patients (88%) showed EBV positivity alone or in combination with HCMV. This observation is of great clinical relevance if we consider that, so far, the majority of papers have instead focused on HCMV infection. Furthermore, the exact correspondence between the macroscopically damaged areas and those carrying the highest viral loads strengthens the hypothesis that both viruses play a role in contributing to mucosal lesions. In this regard, a suggested viral tropism to sites of inflammation[41], and their ability to affect cytokine production may account for how they escape host immune surveillance directly at mucosal level[42]. Moreover, the possible presence of malnourishment and the increasing and early use of aggressive therapies[38] whose main target are T-cells, which play a crucial role in controlling HCMV and EBV latency and reactivation[43], represent potential co-factors in triggering viral colitis. The use of steroids, azathioprine or 6-mercaptopurine, and infliximab, indeed, has been found to produce similar effects in significantly increasing the OR for HCMV infection (3.4, 3.1, and 4.4, respectively), with the combined use of two or three of these drugs yielding an OR of 14.5[2,44]. However, infliximab did not appear to affect the incidence of latent virus reactivation in the short-term[45], but mostly in long-term treatment[3]. Our results only partially confirmed these data, since immunosuppressants did not increase the risk of developing a superimposed viral colitis, while the use of biologic agents and topical steroids was closely related to EBV colitis. Moreover, although anti-tumor necrosis factor-α agents have been shown to cause widespread HCMV infection[46], we did not substantiate this result. Most importantly, as in transplant patients[47], systemic steroid use was identified as a significant risk factor for both HCMV and EBV colitis, probably due to their ability to increase viral protein production, as shown *in vitro*[48]. No additional factors, including duration of therapy or disease, age and degree of malnourishment were found to be related to the risk of developing viral colitis. Interestingly, only those patients with mucosal viral loads approaching 10^5 copies/10^5 cells showed detectable viral DNA even in peripheral blood, as already found in recipients of solid organ transplants where a HCMV end-organ disease may exist independently from systemic involvement[49].

Finally, if our evidence is confirmed by larger studies, the rate of "true" refractoriness to standard therapies may shrink considerably, since a sizeable number of patients would instead be diagnosed as suffering from viral colitis. Remarkably, mucosal healing, mirrored by a sharp decrease in the viral load, was invariably observed in HCMV colitis following specific antiviral therapy[11] together with a quick tapering and then discontinuation of steroids, whilst the immunosuppressive and biological agents may be continued by virtue of their long-lasting effect, which blocks any attempt to recover immunological competence in the short term. By contrast, both the mucosal viral load and indexes of activity remained largely unmodified in those patients with EBV colitis, despite the washout or tapering of the current therapies, and the vast majority of them underwent colectomy. The only patient who showed a substantial, although transient, drop in the mucosal viral load was the one treated with rituximab in an attempt to rapidly deplete the B lymphocytes which host the virus[41], since no efficacious therapy is available to date[4].

In conclusion, the use of quantitative real-time PCR assay on freshly harvested mucosal specimens proved to be more effective than immunohistochemistry in the diagnosis of opportunistic viral infection and allowed us to identify a threshold value that distinguishes infection from end-organ disease, whose clinical management is largely different.

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COMMENTS

Background

Inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis, are disabling, lifelong, pathological conditions affecting the gastrointestinal tract, mainly the colon, triggered and sustained by a dysregulated immune response towards antigens of the gut microbiota. A better understanding of the fine mechanisms responsible for tissue injury has led to the use of more aggressive therapies even in the early phase of the disease aimed at achieving mucosal healing and preventing disease progression. However, the increasing use of immune-suppressant and immune-modulant molecules carries the risk of

opportunistic infections, including those due to human Cytomegalovirus (HCMV) and Epstein Barr virus (EBV).

Research frontiers

To date, the information available on the frequency, role and risk factors of HCMV and EBV infections in IBD exacerbation and their diagnostic and therapeutic approaches is conflicting. The causes for this discrepancy lie in the differences amongst the patients enrolled, the diagnostic methods applied, and the retrospective design of the majority of studies.

Innovations and breakthroughs

This study prospectively investigated the presence of HCMV and EBV infection in IBD patients and control subjects by applying immunohistochemistry and quantitative real-time polymerase chain reaction (PCR) assay, carried out on both peripheral blood and fresh mucosal samples. The latter proved to be the best diagnostic technique, since it allowed us to distinguish between viral colitis and infection by identifying a cutoff value. Interestingly, all refractory IBD patients carried the highest mucosal viral loads, which correlated with the severity of mucosal damage and endoscopic activity. EBV infection was found to be the most prevalent, and steroid therapy was identified as a significant risk factor. Finally, with a view to treatment, a course of antiviral therapy was of benefit in determining both the disappearance of viral DNA and mucosal healing in HCMV-related colitis, whilst the vast majority of patients with EBV colitis underwent colectomy.

Applications

This study contributes to our understanding of the frequency and role of opportunistic viral infection in refractory IBD patients and provides the basis for the use of real time quantitative PCR as the gold standard diagnostic technique in differentiating viral end-organ disease from infection. This technique also allows the patients to be monitored.

Terminology

Inflammatory bowel diseases are chronic enteropathies triggered and sustained by an abnormal immune response to usually-tolerated antigens, which develops in genetically susceptible individuals. Refractoriness is defined as the lack of response to current therapies. Opportunistic viral infections, including HCMV and EBV, are those caused by organisms capable of establishing latency in target cells and reactivating in cases of reduced host defence, such as during immunosuppressive or immunomodulant therapy, giving rise to both systemic and end-organ disease, which can also be localized to the gastrointestinal tract.

Peer-review

The authors investigated CMV and EBV in tissue specimens of refractory and non-refractory mixed IBD patients by quantitative real-time PCR and immunohistochemistry. Additionally, the whole colon was mapped in oder to correlated viral loads to endoscopic lesions. This is a very good paper.

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ORIGINAL ARTICLE

Prospective Study

Acute fatty liver of pregnancy: Over six months follow-up study of twenty-five patients

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Abstract

AIM: To evaluate the prognosis of patients with acute fatty liver of pregnancy (AFLP) 6 mo or longer after discharge.

METHODS: The records of pregnant patients diagnosed with AFLP at Beijing Ditan Hospital over a 16-year period were reviewed in November 2012. Patients were monitored using abdominal ultrasound, liver and kidney functions, and routine blood examination.

RESULTS: A total of 42 patients were diagnosed with AFLP during the study period, and 25 were followed. The mean follow-up duration was 54.5 mo (range: 6.5-181 mo). All patients were in good physical condition, but one patient had gestational diabetes. The renal and liver functions normalized in all patients after recovery, including in those with pre-existing liver or kidney failure. The ultrasound findings were normal in 12 patients, an increasingly coarsened echo-pattern and increased echogenicity of the liver in 10 patients, and mild to moderate fatty liver infiltration in 3 patients. Cirrhosis or liver nodules were not observed in any patient.

CONCLUSION: Acute liver failure and acute renal failure in AFLP patients is reversible. Patients do not require any specific long-term follow-up after recovery from AFLP if their liver function tests have normalized and they remain well.

Key words: Acute fatty liver of pregnancy; Acute liver failure; Acute renal failure; Follow-up study

Core tip: Acute fatty liver of pregnancy (AFLP) is a rare but life-threatening complication of pregnancy. Acute liver failure (ALF) and acute renal failure are the most important and threatening complication of AFLP. ALF due to viral hepatitis is known to potentially cause severe liver fibrosis and cirrhosis, but it is unknown whether the same outcome is possible for AFLP patients. The potential for continued progression or chronic sequelae is also unknown. Hence, the aim of this study was to evaluate the prognosis of patients with AFLP 6 mo or longer after discharge.

Xiong HF, Liu JY, Guo LM, Li XW. Acute fatty liver of pregnancy: Over six months follow-up study of twenty-five patients. *World J Gastroenterol* 2015; 21(6): 1927-1931 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i6/1927.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i6.1927

INTRODUCTION

Acute fatty liver of pregnancy (AFLP) is a rare but life-threatening complication of pregnancy. This condition is defined as microvesicular fatty infiltration of hepatocytes during the second half of pregnancy (usually the third trimester), and it remains a common cause of liver failure during pregnancy. Since it was first described by Stander and Cadden[1] in 1934 and Sheehan[2] in 1940, numerous cases have been reported. The mortality of AFLP used to be very high, up to 85%[3], but with early recognition and prompt termination of pregnancy, the prognosis has improved, and the mortality is now estimated to be between 0% and 12.5%[4-9]. However, AFLP still causes severe maternal morbidity and in some cases mortality, especially in certain critical patients[10].

Acute liver failure (ALF) and acute renal failure (ARF) are the most important and life-threatening complications of AFLP[11,12]. ALF due to viral hepatitis is known to potentially cause severe liver fibrosis and cirrhosis, but it is unknown whether the same outcome is possible for AFLP patients. The potential for continued progression or chronic sequelae is also unknown. The aim of the present study was to evaluate the prognosis of patients with AFLP 6 months or longer after recovery. Notably, this study is the largest single-center, hospital-based follow-up investigation series worldwide.

MATERIALS AND METHODS

Patient selection criteria and exclusion criteria

AFLP was diagnosed based on clinical and laboratory criteria as follows: (1) patients with symptoms of anorexia, fatigue, nausea, vomiting, jaundice, and abnormal liver function during the third trimester of pregnancy; (2) characteristic laboratory findings; (3) ultrasound images showing a fatty liver; (4) exclusion of other causes of liver dysfunction, such as viral hepatitis, biliary tract disease, and cholestasis of pregnancy; and (5) liver biopsy sample with characteristic pathologic changes. All cases conformed to the diagnostic criteria mentioned above, except liver biopsy. Because of their severe condition, prolonged prothrombin times, reduced platelet counts, and/or the patients' refusal, only four patients underwent liver biopsy.

Study design

From January 1996 to May 2012, 42 cases of AFLP were identified at Beijing Ditan Hospital, Capital Medical University in Beijing, China. There were three maternal deaths (7.14%). In the 39 surviving patients, 25 patients consented to follow-up study. Records were reviewed in the 25 enrolled patients for presenting symptoms, laboratory findings, maternal complications, and neonatal outcomes. Demographic data included maternal age, gestational age, and comorbidities. Laboratory evaluation included measurement of liver function, complete blood cell count, coagulation profile, and renal function tests. Patients were monitored using abdominal ultrasound, liver and kidney function tests, and routine blood examination.

Ethics statement

This study was part of the research work "Correlation of mitochondrial trifunctional protein (MTP) α subunit G1528C mutation and AFLP onset" which had been undertaken in May 2011. In accordance with the requirements of the Ethics Committee, we obtained written informed consent from the patients or the authorized clients of the patients (because some patients were in a critical state and were unable to sign the consent themselves). The authorized clients were the husband of the patients; according to Chinese law, they were the next of kin of the patients. We had informed our IRB that we obtained written informed consent from the next of kin of these participants. The Ethics Committee of Beijing Ditan Hospital, Capital Medical University approved the entire study design, methods and consent procedure used in this study. The approval number is QN 2011-06. All the data were collected anonymously.

Definitions

ALF was diagnosed based on clinical and laboratory criteria as follows: evidence of coagulopathy, usually an international normalized ratio $\geqslant$ 1.5 or a prothrombin activity $\leqslant$ 40%; serum total bilirubin $\geqslant$ 171 μmol/L or a daily rise $\geqslant$ 17.1 μmol/L; and any degree of mental alteration (encephalopathy) in a patient without preexisting liver disease and illness < 2 wk duration[13-15].

ARF was defined as the deterioration of renal function over days to weeks and a serum creatinine (Cr) $\geqslant$ 265.2 μmol/L; acute renal impairment was defined as a deterioration in renal function and a Cr > 150 μmol/L. Hypoglycemia was defined as symptoms of hypoglycemia occurring simultaneously with a blood glucose < 50 mg/dL (2.8 mmol/L).

Statistical analysis

Data were analyzed using statistical software (SPSS, Chicago, IL, United States), and $P < 0.05$ was considered statistically significant. Normally distributed data were expressed as the mean and standard deviation, and were analyzed using the *t*-test. Measured data were plotted on a receiver operating

Table 1 Laboratory findings in patients with acute fatty liver of pregnancy during hospitalization and at follow-up

Laboratory test	Peak/nadir average during hospital	Peak/nadir range during hospital	Average at follow-up	Range at follow-up
ALT (U/L)	376.4	34.0-1457.0	17.8	8.2-52.0
AST (U/L)	385.4	10.0-2144.0	18.4	10.1-42.1
TBIL (μmol/L)	258.8	16.0-225.0	12.8	4.7-23.6
Albumin (g/L)	26.0	15.0-37.3	47.2	43.9-54.2
BUN (mmol/L)	9.52	2.0-33.4	5.2	2.9-9.0
Cr (μmol/L)	207.3	66.0-522.0	55.7	45.0-67.7
UA (μmol/L)	331.1	168-542.0	262.5	176.0-390.8
Glucose (mmol/L)	3.5	1.2-6.3	5.8	4.5-16.0
WBC ($\times 10^9$/L)	22.1	4.3-56.3	5.5	3.5-5.5
HGB (g/L)	82.0	37.0-140.8	128.6	102.0-146.0
PLT ($\times 10^9$/L)	81.6	16.0-242.0	226.5	135.0-375.0

characteristic curve and referenced by sensitivity and specificity. Counted data were expressed as the frequency and rate, and χ^2 or Fisher's exact tests were used for analysis. Logistic regression was used to analyze relative factors. Data not normally distributed were expressed as the median and quartile, and analyzed using the rank sum test.

RESULTS

In the 25 enrolled patients, three patients were positive for hepatitis B, which was considered in the differential diagnosis but later excluded. All patients underwent an abdominal ultrasound examination, but a hyperechoic liver was only observed in 15 patients. The diagnosis was confirmed by liver biopsy in only one patient.

During hospitalization, the mean maternal age was 27.2 years (range: 21-34 years), and the mean gestational age at delivery was 35.3 wk (range: 26-42 wk). Delivery occurred by cesarean section in 19 patients and vaginally in six patients. The mean total duration of hospitalization was 25.5 d (range: 6-77 d). The prodromal phase lasted a mean 9 d before admission (range: 0.5-25 d). Reported symptoms included jaundice or dark urine (n = 18), nausea and vomiting (n = 16), malaise (n = 15), and abdominal pain (n = 7). Three patients did not report prodrome and had no complaints related to AFLP at the time of admission.

Laboratory results during hospitalization for the 25 patients are summarized in Table 1. All but one patient had elevated liver enzyme activities; in this particular patient, other criteria for the disease were fulfilled, and the diagnosis was confirmed by liver biopsy at the time of cesarean delivery. The total bilirubin concentration was elevated in 24 cases. Leukocytosis (leukocyte count > 11 000 cells/mm^3) was noted in 23 of 25 patients.

None of the patients underwent liver transplantation. Maternal morbidity was attributed to acute renal injury (18 cases), acute renal failure (8 cases with a urine output < 400 mL/d requiring CRRT), hepatic encephalopathy (10 cases), and postpartum hemorrhage > 500 mL (10 cases). The mean volume of blood loss was 1940 ± 1098 mL (range: 500-4000 mL). Other complications were as follows: hypoglycemia (eight cases), disseminated intravascular coagulopathy (five cases), pneumonia [four cases, all developed acute respiratory distress syndrome (ARDS) requiring mechanical ventilation], mental disturbances such as visual hallucinations, auditory hallucinations, and delusion of persecution (five cases), upper gastrointestinal hemorrhage (two cases), and acute pancreatitis (two cases). There were five cases of diabetes insipidus; all of the patients experienced acute renal injury, but only two patients experienced acute renal failure.

The mean follow-up time duration was 54.5 mo (range: 6.5-181 mo). The laboratory findings are summarized in Table 1. All patients were in good physical condition, but one patient had gestational diabetes. The renal and liver function returned to normal in all patients, including those with pre-existing liver or kidney failure. The ultrasound findings were completely normal in 12 patients; an increased coarse echo-pattern and increased echogenicity of the liver was observed in 10 patients and a mild to moderate fatty liver in 3 patients. Cirrhosis or liver nodules were not observed in any patient.

DISCUSSION

First described in 1934 by Stander and Cadden[1] as "acute yellow atrophy of the liver," AFLP is a medical and obstetric emergency and remains a common cause of liver failure during pregnancy[16]. Recent data indicate a decrease in maternal and neonatal mortalities associated with AFLP owing to increased awareness and earlier recognition of the disease based on its clinical and laboratory findings and significant changes in the facilities over the 16-year recruitment period[6,7,17].

The symptoms presently observed were similar to those found previously[4-9]. Typically, AFLP initially presents with non-specific symptoms such as acute abdominal pain, nausea, vomiting, and malaise, usually occurring during the third trimester of preg-

nancy or during the immediate puerperium. The initial symptoms of AFLP are atypical and could be overlooked, and this disease progresses rapidly and causes multiorgan dysfunction in a very short time; therefore, it is important to be especially vigilant for AFLP development.

After developing the described symptoms, complications typical of AFLP begin to appear, including renal insufficiency, encephalopathy, coma, coagulopathy, infections, ARDS, and acute pancreatitis.

ALF is a common complication of AFLP and one of the most common causes of liver failure during pregnancy. AFLP can sometimes be confused with fulminate liver failure caused by viral hepatitis. In this series, 13 of 25 patients (52%) developed ALF, and nine patients became comatose, which are findings similar to those in other studies[9,18-20]. ALF due to viral hepatitis may result in severe liver fibrosis and cirrhosis, but this does not occur in AFLP patients; we did not observe liver cirrhosis or chronic hepatitis in any of the patients during follow-up, and all have recovered well. Ober and Lecompte[21] reported that the pathologic changes in the liver are "of a reversible kind;" the clinical course and histopathologic findings clearly point to a functional failure-not a destructive form of hepatic insufficiency. Rolfes and Ishak[22] also observed that the lipid infiltration completely disappeared in as little as 5 wk of convalescence and did not progress to hepatic scarring and other chronic sequelae. We suspect that AFLP is usually self-limiting and is a reversible form of acute hepatic failure that does not generally require transplantation. Patients do not require specific long-term follow-up after recovery from AFLP if their liver function tests have normalized and they remain well.

The overall morbidity of renal impairment (Cr > 150 μmol/L) was 72.0%, and incidence of ARF requiring renal replacement treatment was 32.0%. Castro *et al*[9] reported that all AFLP patients had some degree of renal insufficiency on admission. The finding of renal insufficiency early during the course of disease suggests that the renal complications are not the result of hepatic dysfunction. In a study of autopsied patients, Rolfes *et al*[22] demonstrated the presence of sparse microvesicular fat in the kidneys of half the cases in frozen sections stained with oil red-O. The cause of ARF may be related to the inhibition of fat β-oxidation early on[23]; the hepatorenal syndrome probably contributes to the renal dysfunction as well, especially late in the course of disease. Renal function completely recovered at discharge and follow-up, suggesting that the renal damage in AFLP patients is reversible.

In conclusion, acute liver failure and acute renal failure in AFLP patients is reversible. Patients do not require any specific long-term follow-up after recovery from AFLP if their liver function tests have normalized and they remain well.

COMMENTS

Background

Acute fatty liver of pregnancy (AFLP) is a rare but life-threatening complication of pregnancy. Acute liver failure (ALF) and acute renal failure are the most important and threatening complications of AFLP. ALF due to viral hepatitis is known to potentially cause severe liver fibrosis and cirrhosis, but it is unknown whether the same outcome is possible for AFLP patients. The potential for continued progression or chronic sequelae is also unknown.

Research frontiers

In recent years, research has focused on the pathogenesis of AFLP. It is thought to be caused by a disordered metabolism of fatty acids by mitochondria in the mother, caused by deficiency in the long-chain 3-hydroxyacyl-coenzyme A dehydrogenase (LCHAD) enzyme. The gene responsible for LCHAD has been isolated, and the most common mutation found in *AFLP* is the E474Q missense mutation. In another study, the authors also tested for E474Q mutation in these patients and in healthy controls; relevant data have not been published.

Innovations and breakthroughs

This study is the largest single-center, hospital-based, follow-up investigation series worldwide. The study showed that ALF and acute renal failure in AFLP patients is reversible. Patients do not require any specific long-term follow-up after recovery from AFLP if their liver function tests have normalized and they remain well.

Applications

AFLP is a rare but life-threatening complication of pregnancy. This follow-up study can provide a good reference for better understanding of the disease for both patients and doctors.

Peer-review

This is a clearly written manuscript - a small and simple study, materials and methods appear good. The article evaluated the prognosis in patients with acute fatty liver of pregnancy discharged over six months and concluded that acute liver failure and acute renal failure in AFLP patient is reversible; mothers do not need specific long-term follow-up after recovery from AFLP providing their liver function tests have normalized and they remain well.

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ORIGINAL ARTICLE

Prospective Study

Incidence and psychological-behavioral characteristics of refractory functional dyspepsia: A large, multi-center, prospective investigation from China

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Abstract

AIM: To explore the incidence and psychological and behavioral characteristics of refractory functional dyspepsia (RFD) in China.

METHODS: The subjects of this study were 1341 new outpatients with functional dyspepsia (FD) who were diagnosed according to the Rome Ⅲ criteria at four hospitals in Guangdong Province between June and September 2012, and 100 healthy volunteers. All subjects completed questionnaires and scales administered.

RESULTS: Three-hundred and twenty-seven of the 1341 patients with FD had RFD (24.4%). Patients with RFD had a longer disease duration and a more severe form of the disease than patients with non-refractory FD (NRFD). The prevalence of depression and anxiety symptoms was higher in patients with RFD than in patients with NRFD. The prevalence of unhealthy eating behaviors, lack of physical activity, and sleeping disorders was higher in patients with RFD than in patients with NRFD. Patients with RFD sought medical advice on more occasions and spent more money on treatment than patients with NRFD. Finally, patients with RFD had poorer quality of life than patients with NRFD.

CONCLUSION: RFD is not rare in clinical practice and should get attention by patients and doctors because of its long duration, severe symptoms, and associations with abnormal psychology and poor quality of life.

Key words: Refractory functional dyspepsia; Psychological-behavioral characteristics; Depression; Anxiety; Behavior; Quality of life

Core tip: Functional dyspepsia is the most common functional gastrointestinal disease. Refractory functional dyspepsia (RFD) even makes patients see a doctor repeatedly and aggravates their medical expenses. However, there is rare research concerning the psychological-behavioral characteristics of RFD patients until now. Thus we performed a large, multicenter investigation of RFD in China, and the findings may illustrate the importance of recognition and diagnosis of RFD, and provide a basis for clinical treatment and the relapse prevention of this condition.

Jiang SM, Jia L, Lei XG, Xu M, Wang SB, Liu J, Song M, Li WD. Incidence and psychological-behavioral characteristics of refractory functional dyspepsia: A large, multi-center, prospective investigation from China. *World J Gastroenterol* 2015; 21(6): 1932-1937 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i6/1932.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i6.1932

INTRODUCTION

Functional dyspepsia (FD) is one of the most common functional gastrointestinal diseases[1]. Because of differences between Asian and Western countries in diet, lifestyle, environment, and genetic factors, functional gastrointestinal diseases in Asian countries are different from those in Western countries with regard to symptoms, epidemiology, etiology, and pathogenesis[1,2]. Refractory FD (RFD) refers to FD that has continuous symptoms for at least 6 mo that has been unresponsive to at least two medical treatments, such as acid suppressors, proton pump inhibitors, prokinetics, or *Helicobacter pylori* (*H. pylori*) eradication[3]. The failure to respond to treatments is the main reason that RFD patients make repeated visits to a doctor and escalate their medical expenses. However, the pathogenesis of RFD and the reasons this disease fails to respond to treatment are not fully understood. Moreover, there is little research concerning the psychological and behavioral characteristics of patients with RFD.

In this study, we present data from a large, multicenter investigation of RFD in China that were collected as a part of a registered study (ChiCTR-TRC-12001968). The focus of the present study was to quantify the incidence of RFD and to identify the psychological and behavioral characteristics of patients with RFD to illustrate the importance of recognizing and diagnosing RFD, and to provide a basis for clinical treatment and relapse prevention.

MATERIALS AND METHODS

Ethics statement

All participants gave written informed consent. The study was approved by the ethics committees of Guangzhou Nansha Central Hospital, Guangzhou First People's Hospital, Guangdong Province Second People's Hospital, and Meizhou Municipal People's Hospital.

Study subjects

The study involved 9802 patients who visited the digestive department outpatient service of Guangzhou Nansha Central Hospital, Guangzhou First People's Hospital, Guangdong Province Second People's Hospital, or Meizhou Municipal People's Hospital from June to September 2012. FD and the subtype of FD [epigastric pain syndrome (EPS) or postprandial distress syndrome (PDS)] were diagnosed according to the Rome Ⅲ criteria[4]. Patient exclusion criteria were: (1) an endoscopic examination revealed an ulcer, erosion, tumor, another organic disease, or evidence of esophagitis; (2) a history of ulcers, erosion, tumors, other organic diseases, or esophagitis; (3) a laboratory examination, ultrasound, or X-ray revealed an organic disease associated with the liver, gallbladder, pancreas, or intestine; (4) diabetes, connective tissue disease, mental disease, or other systemic diseases; (5) a history of abdominal operations; (6) pregnancy or lactation; and (7) other functional gastrointestinal disorders such as irritable bowel syndrome and gastroesophageal reflux disease. In addition, the FD patients who had to report continuous symptoms for at least 6 mo but failed to respond to at least two medical treatments (such as acid suppressors, proton pump inhibitors, prokinetics, or *H. pylori* eradication) were defined with RFD[3]. All FD patients who did not meet these criteria were classed as non-refractory FD (NRFD) patients.

One hundred healthy volunteers were included as control subjects. Healthy volunteers did not exhibit the following symptoms for 3 mo prior to the study: postprandial fullness, early satiety, epigastric pain, burning epigastric pain, abdominal pain, diarrhea, constipation, and other gastrointestinal symptoms. Healthy volunteers had normal function of the heart, liver, and kidneys, no recent history of drug use, no history of mental disease and no history of surgery.

Data collection

Two unified training doctors obtained data from patients and healthy volunteers in all hospitals. Data collection took place *via* face-to-face interviews in a quiet environment. Consultation time was 20-30 min per subject.

The 25-item Nepean Dyspepsia Index was utilized to evaluate the severity of patients' symptoms. The symptom checklist included frequency, severity and bothersomeness of 15 upper gastrointestinal symptoms, and a higher total score indicates a more severe digestive symptom[5].

The 17-item Hamilton Depression Rating Scale (HAMD) and the 14-item Hamilton Anxiety Rating

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

Feature	RFD (*n* = 327)	NRFD (*n* = 1014)	Healthy volunteers (*n* = 100)	*P* value[1]	*P* value[2]
Gender				0.300	0.470
Male	127 (38.8)	427 (42.1)	45 (45.0)		
Female	200 (61.2)	587 (57.9)	55 (55.0)		
Age group				0.110	0.110
≤ 20 yr	8 (2.4)	39 (3.8)	2 (2.0)		
21-30 yr	54 (16.5)	215 (21.2)	24 (24.0)		
31-40 yr	84 (25.7)	289 (28.5)	28 (28.0)		
41-50 yr	113 (34.6)	294 (29.0)	27 (27.0)		
51-60 yr	52 (15.9)	130 (12.8)	17 (17.0)		
> 60 yr	16 (4.9)	47 (4.6)	2 (2.0)		
Age, yr	41.91 ± 11.30	39.60 ± 11.81	39.83 ± 12.42	0.001	0.780
Subtype					
EPS	120 (36.7)	457 (45.1)	-	0.010	-
PDS	94 (28.7)	280 (27.6)	-	0.690	-
EPS + PDS	113 (34.6)	277 (27.3)	-	0.010	-
Duration of disease, (yr)	6.01 ± 2.87	3.76 ± 1.83	-	0.002	-
Severity of disease					
Mild	101 (30.9)	459 (45.3)	-	0.001	-
Moderate	148 (45.3)	407 (40.1)	-	0.100	-
Severe	78 (23.9)	148 (14.6)	-	0.003	-

[1]Comparison between RFD and NRFD patients; [2]Comparison between all functional dyspepsia patients (RFD and NRFD combined) and healthy volunteers. Continuous data were compared across groups using one-way ANOVA and Student-Newman-Keuls test for multiple comparisons. Count data were compared across groups using a χ^2 test. RFD: Refractory functional dyspepsia; NRFD: Non-refractory functional dyspepsia; EPS: Epigastric pain syndrome; PDS: Postprandial distress syndrome.

Scale (HAMA) were used to evaluate the degrees of depression and anxiety symptoms. Higher scores indicate more severe depressive or anxious conditions. Depression symptoms were classified as severe (HAMD score ≥ 25), moderate (HAMD score 18-24), mild (HAMD score 7-17) or none (HAMD score ≤ 6)[6,7]. Anxiety symptoms were also classified as severe (HAMA score ≥ 24), moderate (HAMA score 15-23), mild (HAMA score 8-14) or none (HAMA score ≤ 7)[8,9].

The unhealthy eating behaviors were investigated including the aspects of skip meals (less than three meals a day), eating late (1 h later than the normal meal time) and eating extra meal (more than three meals a day). The working behaviors included the aspects of working in the day (the time period from 6 am to 6 pm), working at night (the time period from 6 pm to 6 am) and no work, and study was also considered a work. Physical activity was evaluated using the International Physical Questionnaire (IPAQ), and low/moderate/high levels of physical activity were defined according to the guideline for data analysis of IPAQ[10]. Sleeping was also assessed using the Pittsburgh Sleep Quality Index (PSQI). A high PSQI score indicates poor sleep, and patients with a PSQI score > 5 were considered to have a sleep disorder[11]. Quality of life was evaluated using the Short Form 36 Health Survey Questionnaire (SF-36). High SF-36 scores indicate better quality of life[12]. Patients were investigated about the amount of money spent on former treatments (drugs, examination, fee, traffic, *etc*.) when they were diagnosed with FD or RFD. The money was quantified by Chinese Yuan (CNY).

Statistical analysis

Statistical analyses were performed with SPSS 13.0 for Windows. Continuous data are expressed as mean ± SD, and were compared across groups (RFD, NRFD, healthy volunteers) using one-way ANOVA and Student-Newman-Keuls test for multiple comparisons. Count data were compared across groups using a χ^2 test. All tests were two-tailed and $P < 0.05$ was considered statistically significant. Especially, a χ^2 test for independence was used to determine whether severity of disease, depression and anxiety symptoms was related to RFD, and $\chi^2 > 6.635$ was considered statistically significant.

RESULTS

Study sample

Of 1600 patients diagnosed with FD, 390 fulfilled the criteria for RFD. A total of 1341 patients (n = 327 for RFD and n = 1014 for NRFD) completed the questionnaire battery. These patients comprised 554 males and 787 females and were between 18 and 76 years old (mean age = 40.17 ± 11.72 years). The 100 healthy volunteers comprised 45 males and 55 females and were between 18 and 78 years old (mean age = 39.83 ± 12.42 years). Characteristics of healthy volunteers were similar to those of the patients (Table 1).

Incidence and disease characteristics

Of 9802 outpatients, 1600 (16.3%) were diagnosed with FD and 390 (4.0%) were diagnosed with RFD.

Table 2 Psychological and behavioral characteristics and quality of life of the study subjects *n* (%)

Characteristic	RFD (*n* = 327)	NRFD (*n* = 1014)	Healthy volunteers (*n* = 100)	*P* value
Depression symptoms	207 (63.3)[a, c]	212 (20.9)[c]	10 (10.0)	0.002
Mild	112 (34.3)[a, c]	170 (16.8)	10 (10.0)	0.001
Moderate	61 (18.7)[a, c]	34 (3.4)	0 (0)	0.001
Severe	34 (10.4)[a, c]	8 (0.8)	0 (0)	0.004
HAMD score	11.65 ± 6.51[a, c]	7.15 ± 3.34[c]	1.66 ± 2.50	0.003
Anxiety symptoms	201 (61.5)[a, c]	236 (23.3)[c]	10 (10.0)	0.002
Mild	97 (29.7)[a, c]	162 (16)	10 (10.0)	0.001
Moderate	74 (22.6)[a, c]	60 (5.9)[c]	0 (0)	0.003
Severe	30 (9.2)[a, c]	14 (1.4)	0 (0)	0.001
HAMA score	10.84 ± 5.82)[a, c]	6.83 ± 3.53)[c]	2.26 ± 2.68	0.001
Both depression and anxiety symptoms	186 (56.9)[a, c]	171 (16.9)[c]	6 (6.0)	0.001
Eating behavior				
Skip meals	68 (20.8)[a, c]	131 (12.9)[c]	4 (4.0)	0.010
Eating late	92 (28.1)[a, c]	222 (21.9)[c]	6 (6.0)	0.001
Eating extra meal	30 (9.2)[a, c]	55 (5.4)[c]	3 (3.0)	0.010
Physical activity level				
High	98 (30.0)[c]	332 (32.7)[c]	44 (44.0)	0.030
Moderate	156 (47.7)	536 (52.9)	54 (54.0)	0.240
Low	73 (22.3)[a, c]	146 (14.4)[c]	2 (2.0)	0.001
Working time				
Working in the day	160 (48.9)	529 (52.2)	56 (56.0)	0.400
Working at night	22 (6.7)	101 (10.0)[c]	2 (2.0)	0.010
No work	126 (38.5)[b]	333 (32.8)	25 (25.0)	0.030
Sleeping disorder	204 (62.4)[a, c]	334 (32.9)[c]	13 (13.0)	0.001
PSQI score	8.87 ± 5.00[a, c]	5.70 ± 4.12[c]	2.95 ± 2.78	0.001
SF-36 score	61.17 ± 16.77[a, c]	74.96 ± 13.51[c]	88.18 ± 7.30	0.001
Number of times seeking medical advice	13.41 ± 7.66	4.09 ± 2.20	-	0.002
Cost of treatments	3797.28 ± 1406.41	1523.07 ± 665.35	-	0.001

Continuous data are expressed as mean ± SD, and were compared across groups using one-way ANOVA and Student-Newman-Keuls test for multiple comparisons. Count data were compared across groups using a χ^2 test. [a]$P < 0.05$ *vs* NRFD patients; [c]$P < 0.05$ *vs* healthy volunteers. RFD: Refractory functional dyspepsia; NRFD: Non-refractory functional dyspepsia; HAMA: Hamilton Anxiety Scale; HAMD: Hamilton Depression Scale; PSQI: Pittsburgh Sleep Quality Index; SF-36: MOS 36-item Short Form Health Survey.

RFD accounted for 24.4% (390/1600) of all FD diagnoses. Among the 1341 patients who completed the questionnaire, the incidence of RFD was highest in patients that were 41-50 years old. Females were more than males in patients with RFD (61.2% *vs* 38.8%, $P < 0.05$). Incidence of the EPS subtype was lower in patients with RFD than in patients with NRFD (36.7% *vs* 45.1%, $P < 0.05$), whereas incidence of the EPS + PDS subtype was higher in patients with RFD than in patients with NRFD (34.6% *vs* 27.3%, $P < 0.05$; Table 1). Disease duration was longer for patients with RFD than for patients with NRFD (6.01 ± 2.87 years *vs* 3.76 ± 1.83 years, $P < 0.05$; Table 1). RFD was related to severity of disease ($\chi^2 > 6.635$), most patients with RFD or NRFD had moderate symptoms, but the proportion of patients with severe symptoms was higher in RFD than in NRFD (23.9% *vs* 14.6%, $P < 0.05$; Table 1).

Psychological characteristics

The prevalence of depression and anxiety symptoms was higher in patients with RFD (63.3%, 207/327 and 61.5%, 201/327, respectively) than in patients with NRFD (20.9% and 23.3%, respectively) or healthy volunteers (10.0% and 10.0%, respectively; Table 2). Similarly, more patients presented both depression and anxiety symptoms in the RFD group than those in the NRFD group or healthy volunteers group ($P < 0.05$ for all; Table 2). This trend was apparent for all degrees of severity. The prevalence of mild, moderate, and severe depression and anxiety symptoms was higher in patients with RFD than in patients with NRFD ($P < 0.05$ for all; Table 2). Moreover, RFD was measured related to severity of depression and anxiety symptoms ($\chi^2 > 6.635$ for both). The total HAMA and HAMD scores, which represent the severity of depression and anxiety symptoms, respectively, were also higher in patients with RFD than in patients with NRFD and healthy volunteers ($P < 0.05$ for all; Table 2).

Behavioral characteristics

The prevalence of unhealthy eating behaviors, such as skipping meals, eating late, and eating extra meals, was higher in patients with RFD than in patients with NRFD or healthy volunteers ($P < 0.05$ for all; Table 2). Most patients and healthy volunteers reported a moderate level of physical activity, but the proportion of subjects that reported a low level of exercise was higher among patients with RFD than patients with NRFD or healthy volunteers ($P < 0.05$; Table 2). The proportion of subjects without work was higher

among patients with RFD than patients with NRFD or healthy volunteers, but a significant difference only existed between RFD patients and healthy volunteers (Table 2). The proportion of subjects who worked during the day was similar among the three groups. The proportion of subjects who worked at night was higher in patients with NRFD than in patients with RFD or healthy volunteers, but a significant difference only existed between NRFD patients and healthy volunteers (Table 2). The prevalence of sleeping disorders was higher in patients with RFD (62.4%, 204/327) than in patients with NRFD (32.9%, 334/1014, $P < 0.05$) or healthy volunteers (13.0%, 13/100, $P < 0.05$), and PSQI scores, which represent quality of sleep, were higher in patients with RFD than in patients with NRFD or healthy volunteers ($P < 0.05$ for both; Table 2).

Quality of life

The SF-36 score, which indicates quality of life, was lower in patients with RFD than in patients with NRFD or healthy volunteers ($P < 0.05$ for both; Table 2). Furthermore, the numbers of times that subjects had sought medical advice and the amount of money spent on treatment were higher in patients with RFD than in patients with NRFD ($P < 0.05$ for both; Table 2).

DISCUSSION

Estimates of the worldwide prevalence of dyspepsia in the general population range from 7%-40%[13]. With a decline in the incidence of peptic ulcer disease and gastric cancer, the incidence of FD is set to increase. RFD has a poor curative effect[3,14,15], thus it should be taken seriously by the medical community. In 2000, Hamilton *et al*[3] defined chronic FD as FD that had been present for at least half a year and persisted after at least two conventional drug treatments. At present, there is no unified and precise definition of RFD. In this study, RFD was defined according to both the Rome Ⅲ criteria and the criteria of Hamilton *et al*[3], and accounted for 4.0% of all digestive department outpatients and 24.4% of FD cases, indicating that RFD is not rare in clinical practice. RFD was characterized by a long course and high disease severity.

EPS is the main subtype of FD in Western countries, but PDS is the predominant subtype in Japan[16]. Differences in the prevalence of FD subtypes may be uniquely related to differences in dietary habits, customs, the social and cultural environment, and genetic factors. In this study, we found that the prevalence of the EPS + PDS subtype was significantly higher among patients with RFD than among patients with NRFD. This may provide a clue as to the obstinacy of RFD.

The pathogenesis of functional gastrointestinal disorders is complex, and is related to gastrointestinal motility dysfunction, visceral hypersensitivity[17-19], the change between brain-gut axis and gastrointestinal hormones, eating behaviors[20-24], or psychology[25]. Doi *et al*[26] reported that the prevalence of psychological disorders was significantly higher in patients with FD than in the general population. Similarly, the psychological outcomes of our study indicated that the prevalence of depression and anxiety symptoms was higher in patients with RFD than in patients with NRFD or healthy volunteers, and the severity of symptoms associated with depression and anxiety was higher in patients with RFD than in patients with NRFD or healthy individuals.

Some behavioral characteristics were also revealed in this investigation. The prevalence of unhealthy eating behaviors (such as meal skipping, eating late, and eating an extra meal), low levels of physical activity, no work, and sleeping disorders was higher for patients with RFD than for patients with NRFD or healthy volunteers. Sleeping disorders are common for patients with FD, both in China and around the world. Miwa[23] reported that the proportion of subjects who thought they slept enough was significantly lower among patients with FD-irritable bowel syndrome overlap than among control subjects. We believe that sleep disorders in RFD may be the result of other underlying problems, such as depression, anxiety, or other psychological conditions.

Because of the characteristics described above, RFD patients sought medical advice more often and spent more money on treatment than patients with NRFD or healthy volunteers. All of these negative factors combine to reduce quality of life, which was reflected in our SF-36 results.

In summary, the medical community should pay more attention to RFD diagnosis and to the importance of an unhealthy lifestyle, abnormal psychological characteristics, and sleeping disorders in RFD.

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COMMENTS

Background

The incidence of functional dyspepsia (FD) is set to increase nowadays. Refractory functional dyspepsia (RFD) even makes patients see a doctor repeatedly and aggravate their medical expenses. However, the pathogenesis of RFD and the reasons this disease fails to respond to treatment are not fully understood. There is rare research concerning the psychological-behavioral characteristics of RFD patients until now.

Research frontiers

At present, there is no unified and precise definition of RFD. The research about pathogenesis, psychological and behavioral characteristics of RFD is limited.

Innovations and breakthroughs

This study was to quantify the incidence of RFD and to identify the psy-

chological and behavioral characteristics of patients with RFD by a large, multi-center and prospective investigation. The findings revealed that RFD is not rare in clinical practice and should get attention by patients and doctors because of its long duration, severe symptoms, and associations with abnormal psychology and poor quality of life.

Applications

The findings can illustrate the importance of recognizing and diagnosing RFD, and to provide a basis for clinical treatment and relapse prevention.

Terminology

Epigastric pain syndrome or postprandial distress syndrome is one of the subtypes of FD. It is detailedly defined in the Rome Ⅲ criteria.

Peer-review

This is a good and practical study in which the authors analyzed the incidence and psychological-behavioral characteristics of RFD patients. It is believed that the findings can enhance the recognition and diagnosis for RFD, and provide a basis for the further research about RFD.

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ORIGINAL ARTICLE

Randomized Controlled Trial

Split-dose menthol-enhanced PEG *vs* PEG-ascorbic acid for colonoscopy preparation

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Abstract

AIM: To compare the efficacy and palatability of 4 L polyethylene glycol electrolyte (PEG) plus sugar-free menthol candy (PEG + M) *vs* reduced-volume 2 L ascorbic acid-supplemented PEG (AscPEG).

METHODS: In a randomized controlled trial setting, ambulatory patients scheduled for elective colonoscopy were prospectively enrolled. Patients were randomized to receive either PEG + M or AscPEG, both split-dosed with minimal dietary restriction. Palatability was assessed on a linear scale of 1 to 5 (1 = disgusting; 5 = tasty). Quality of preparation was scored by assignment-blinded endoscopists using the modified Aronchick and Ottawa scales. The main outcomes were the palatability and efficacy of the preparation. Secondary outcomes included patient willingness to retake the same preparation again in the future and completion of the prescribed preparation.

RESULTS: Overall, 200 patients were enrolled (100 patients per arm). PEG + M was more palatable than AscPEG (76% *vs* 62%, $P = 0.03$). Completing the preparation was not different between study groups (91% PEG + M *vs* 86% AscPEG, $P = 0.38$) but more patients were willing to retake PEG + M (54% *vs* 40% respectively, $P = 0.047$). There was no significant difference between PEG + M *vs* AscPEG in adequate cleansing on both the modified Aronchick (82% *vs* 77%, $P = 0.31$) and the Ottawa scale (85% *vs* 74%, $P = 0.054$). However, PEG + M was superior in the left colon on the Ottawa subsegmental score (score 0-2: 94% for PEG + M *vs* 81% for AscPEG, $P = 0.005$) and received significantly more excellent ratings than AscPEG on the modified Aronchick scale (61% *vs* 43%, $P = 0.009$). Both preparations performed less well in afternoon *vs* morning examinations (inadequate: 29% *vs* 15.2%, $P = 0.02$).

CONCLUSION: 4 L PEG plus menthol has better palatability and acceptability than 2 L ascorbic acid-PEG and is associated with a higher rate of excellent

preparations; Clinicaltrial.gov identifier: NCT01788709.

Key words: Colonoscopy; Bowel preparation; Efficacy; Tolerability; Menthol

Core tip: Colon preparations are generally poorly tolerated. As a result, suboptimal bowel preparation can occur in as many as 25%-40% of cases. Volume and palatability of the purgative are important determinants of tolerability and adherence and, consequently of efficacy. In this randomized controlled trial, we investigate the efficacy and palatability of two colonic preparations (4 L PEG + menthol candy *vs* 2 L ascorbic acid supplemented -PEG) given as split-dose and with minimal dietary restrictions. Both preparations were similarly effective at achieving adequate colon preparation but 4 L PEG + M had superior palatability and tolerability and was associated with more excellent ratings.

Sharara AI, Harb AH, Sarkis FS, Chalhoub JM, Badreddine R, Mourad FH, Othman M, Masri O. Split-dose menthol-enhanced PEG *vs* PEG-ascorbic acid for colonoscopy preparation. *World J Gastroenterol* 2015; 21(6): 1938-1944 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i6/1938.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i6.1938

INTRODUCTION

Colorectal cancer continues to be one of the leading causes of death worldwide[1]. A combination of early detection and removal of precursor lesions has proven beneficial in decreasing its incidence and mortality[2,3]. However and despite the advent of several screening modalities[4-7], optical colonoscopy remains the preferred procedure due to its superior diagnostic sensitivity[8], unique capability that allows sampling and removal of luminal pathology[9], and cost-effectiveness[10]. These performance characteristics are, however, highly dependent on the quality of colon preparation[11,12]. Suboptimal preparations are associated with a higher adenoma miss rate, prolonged procedure time, incomplete examinations[11], and increased cost due to need for an earlier repeat examination[13].

Polyethylene glycol electrolyte (PEG) solution is the most commonly used purgative for colon cleansing due to its superior safety profile[14] and high efficacy[15]. In a recent meta-analysis, 4 L split-dose PEG was found to be superior to other bowel preparation comparators suggesting it should be the standard against which new bowel preparations should be compared[15]. However, the unpalatable taste and large volume required for proper cleansing are the most commonly reported reasons to avoid colonoscopy[12]. The preparation is poorly tolerated by patients resulting in incomplete adherence and consequently low-quality preparations[16]. An important development was the concept of dose splitting, where as much as half of the preparation is consumed on the day of the examination, leading to improved efficacy[17] and tolerability[18]. Further refinements[19-21], adjuvants[18,22], and modifications[23-25] of PEG solutions followed. The addition of high-dose ascorbic acid to PEG (AscPEG) improved taste and helped reduce the volume of the preparation to 2 L, making it one of the most commonly prescribed purgatives in the United States. Another simple refinement involves the addition of menthol candy to 4 L split PEG resulting in significant benefit in terms of patient tolerability, acceptability and compliance, and leading to a higher rate of excellent preparations[26]. In this study, we aim to directly compare two tested modifications of the split-dose PEG preparation, namely 4 L PEG with menthol candy (PEG + M) *vs* 2 L AscPEG.

MATERIALS AND METHODS

Patients

The study was conducted at the American University of Beirut Medical Center between February and December 2013. Patients seen in the outpatient clinics requiring elective colonoscopies were approached about the study by their respective endoscopist. Exclusion criteria included age less than 18, pregnant or lactating women, prior intestinal resection or bariatric surgery, chronic renal disease (creatinine clearance < 60 mL/min), severe congestive heart failure (New York Heart Association class Ⅲ or Ⅳ), dehydration, history of severe constipation (< 1 bowel movement every 3 d), chronic laxative abuse, and history of inflammatory bowel disease. Patients with phenylketonuria, known significant gastroparesis, known or suspected bowel obstruction were also excluded. Due to potential for priming by a previous colonoscopy experience in the past 5 years that might alter anticipation and response, patients who had a colonoscopy within the last 5 years were also excluded.

After informed consent was obtained, patients were referred to the study coordinator where they received one of the two regimens based on a previously computer-generated 1:1 randomization list. Detailed written instructions and verbal explanations were provided to all patients. On the day of the colonoscopy, patients were interviewed by an independent investigator prior to the procedure. Colonoscopies were performed by one of five endoscopists blinded to the preparation. All exams were performed under conscious sedation in the endoscopy unit between 9:00 am and 4:00 pm. The study protocol was approved by the Institutional Review Board of the American University of Beirut on December 2012, and the

study was registered with Clinicaltrial.gov identifier: NCT01788709.

Preparation instructions

Detailed written instructions and verbal explanations were provided to all patients at the time of colonoscopy scheduling, emphasizing the importance of complete intake of the solution to ensure a more effective procedure. On the day preceding the colonoscopy, patients were allowed to consume an unrestricted breakfast and lunch till 3 pm, followed by a full-fluid dinner (*e.g.*, milk, water, soda, clear broth, tea, or yoghurt) until 7 pm. Only clear fluids were allowed after 7 pm (*e.g.*, water, clear soda, tea).

Patients in the first arm received 4 L PEG (Fortrans®, Ibsen, Paris, France) divided into 2 L consumed from 7-9 pm on the day preceding the colonoscopy, and 2 L on the day of the colonoscopy, taken no earlier than 4 h before the scheduled appointment, and completed a minimum of 90 min before the procedure. Patients in this arm were provided with sugar-free, colorless, menthol candy (Halls®, Cadbury Adams, NJ, United States) and instructed to suck on a candy while drinking the PEG solution (PEG + M). Patients in the second arm received 2 L reduced-volume ascorbic acid-supplemented PEG-electrolyte solution (AscPEG) (MoviPrep®, Norgine, Harefield, United Kingdom) mixed according the manufacturer's instructions plus 1L of clear fluids of the patient's choice and dose-split over 2 d. The first liter of AscPEG was consumed at 7 pm the day before colonoscopy with a minimum of 500 mL of clear fluids, and another 1 L of AscPEG plus a minimum of 500 mL of clear fluids on the day of the colonoscopy, to be completed no more than 4 h before the procedure and at least 90 min before the procedure.

Data collection

Immediately before colonoscopy, patients were interviewed by an independent investigator. Patients were asked to report their perception of the solution's palatability by checking a linear scale on a boxed diagram from 1 to 5 (disgusting, moderately poor taste, slightly poor taste, acceptable, and tasty) and express the degree of willingness to take the preparation again in the future. An assessment of compliance and adherence was also performed with the volume remaining of the solution recorded. The quality of bowel cleansing was graded immediately following colonoscopy by the performing endoscopist who was blinded to the preparation assigned. Endoscopists were asked to provide a score for each patient using the modified Aronchick scale and Ottawa scale.

Study design and endpoints

The primary end points of the study were the quality of the preparation and the palatability of the administered solution. Secondary endpoints included willingness to retake the same preparation again in the future, and completion of the preparation. The solutions were considered palatable if they received 4 or 5 on the 5-point palatability scale (acceptable or tasty) and unpalatable for a score of 1-3 (disgusting, moderately poor taste, or slightly poor taste). Patients with an Aronchick score of excellent or good were considered to have an adequate preparation, whereas those with poor, inadequate, and fair were considered to have an inadequate preparation. On the Ottawa scale, segmental scores were collected and an overall score was calculated. A total score of > 8 was considered to indicate an inadequate preparation and a score of $\leqslant$ 7 was deemed adequate. Preparations with a total score of $\leqslant$ 4 were considered excellent, provided that no individual colonic segment received a score higher than 1. Patients were considered to have completed the preparation if they consumed $\geqslant$ 90% of the preparation volume.

Sample size calculation was based on published data showing 90% adequate preparation with menthol-enhanced PEG (PEG + M)[26] and 65%-85% (average: 75%) for reduced volume AscPEG[24,27,28]. Using α of 0.05 and a power of 0.80, the sample size required to show significance was calculated to be 97 patients per arm. Hence, it was decided to recruit 100 patients per arm taking into account possible withdrawals. SPSS version 20.0 (SPSS Inc., Chicago, Illinois, United States) was used for data entry and analysis. The proportions in 2 × 2 contingency tables were compared by the χ^2 test. A *P* value of less than 0.05 was considered significant. The primary investigator and co-authors had access to the study data and had reviewed and approved the final manuscript.

RESULTS

Two hundred patients successfully completed the study with 100 patients enrolled in each arm. Nine patients cancelled appointments for reasons unrelated to the quality or taste of the preparation and were replaced by a similar number of patients. Additionally, 4 patients took a different preparation or unauthorized adjuvants, 3 patients were found to have IBD at the time of colonoscopy, 1 patient forgot to use the menthol candy, 1 patient was found to have a history of laxative abuse, and 1 patient could not be scoped beyond the left colon. All of these cases were excluded. Two patients both belonging to the AscPEG arm had cancelled procedure due to bad preparation quality. These patients were included in the study and received the worst scoring on both scales. The mean age of enrolled patients was 54.5 ± 13.7 years (range: 21-85) with 52% males. Patients' characteristics and indications for colonoscopy were not different between the two groups (Table 1).

There was no difference between the two pre-

Table 1 Patient characteristics

	PEG + M	AscPEG	*P* value
Age (mean ± SD, yr)	55 ± 13.8	54 ± 13.7	0.88
Male subjects	54%	50%	0.74
BMI (mean ± SD, kg/m^2)	27 ± 4.7	27 ± 4.9	0.54
Indication			
Screening	46%	43%	0.70
Hematochezia	13%	13%	0.95
Abdominal pain	14%	12%	0.75
Change in bowel habits	10%	6%	0.41
Surveillance	7%	9%	0.53
Positive FOBT	4%	5%	0.74
Weight loss	2%	1%	0.27
Anemia	2%	5%	0.31
Diverticular disease	0%	2%	0.20
Others	2%	4%	0.28

PEG + M: PEG electrolyte solution + menthol; AscPEG: Ascorbic acid PEG electrolyte solution.

Table 2 Percentage adequate preparation by colonic segment using the Ottawa scale

	PEG + M	AscPEG	*P* value
Right	84%	79%	0.340
Mid	95%	89%	0.110
Left	94%	81%	0.005[1]
Overall	85%	74%	0.054

[1]Significant *P*-value. PEG + M: PEG electrolyte solution + menthol; AscPEG: Ascorbic acid PEG electrolyte solution.

parations in the rate of adequate preparations on the Aronchick scale (82% for PEG + M *vs* 77% for AscPEG, $P = 0.31$) (Figure 1). On the same scale, a significantly higher number of patients receiving PEG + M were found to have an excellent preparation (61% *vs* 43% AscPEG, $P = 0.009$). The mean score on the Ottawa scale was better for PEG + M *vs* AscPEG (5.1 ± 2.4 *vs* 5.8 ± 3.0, $P = 0.09$) as was the rate of adequate preparations (overall score $\leqslant$ 7: 85% *vs* 74%, $P = 0.054$) but these were not statistically significant (Figure 2). Table 2 shows the Ottawa scores according to colonic segment. Using a segmental score of 0-2 as indication of an adequate cleansing[29] there was no significant difference between preparations in the right and mid-colon. However, PEG + M was superior in the left colon (94% for PEG + M *vs* 81% for AscPEG, $P = 0.005$).

PEG + M was significantly more palatable than AscPEG (76% *vs* 62%, $P = 0.03$) (Figure 3). Similarly, a significantly higher number of patients were willing to retake PEG + M again in the future compared to AscPEG (54% *vs* 40%, $P = 0.047$). Patients in the PEG + M arm had a lower rate of non-compliance with the prescribed volume compared to AscPEG, but this difference was not significant (9% *vs* 14%, $P = 0.38$).

There was no difference in the number of patients undergoing colonoscopy in the afternoon between

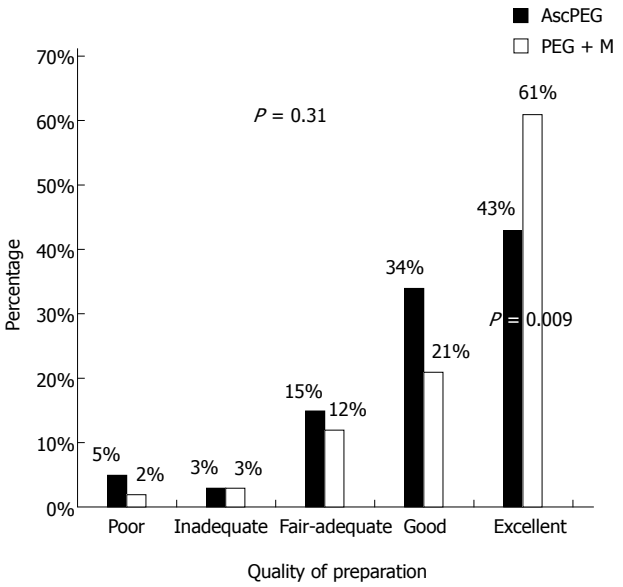

Figure 1 Quality of bowel preparation on the modified Aronchick scale. *P*-value for the difference between AscPEG and PEG + M Aronchick scores is 0.31; *P*-value for the difference between excellent results with PEG + M and AscPEG is 0.009. PEG + M: PEG electrolyte solution + menthol; AscPEG: Ascorbic acid PEG electrolyte solution.

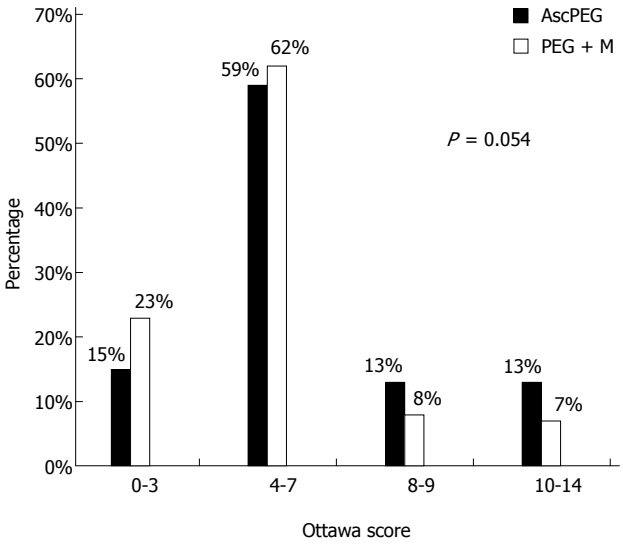

Figure 2 Overall preparation score according to the Ottawa score (a lower score indicates a better preparation). *P*-value for the difference between AscPEG and PEG + M Ottawa scores is 0.054. PEG + M: PEG electrolyte solution + menthol; AscPEG: Ascorbic acid PEG electrolyte solution.

study groups (40% PEG + M *vs* 35% AscPEG, P = NS). However, PM colonoscopies had a higher rate of inadequate preparation compared to AM colonoscopies (29% *vs* 15.2%, $P = 0.02$) with no significant difference between study groups. On univariate analysis, BMI, age, and gender were not associated with inadequate preparations.

DISCUSSION

The ideal bowel preparation should be simple to

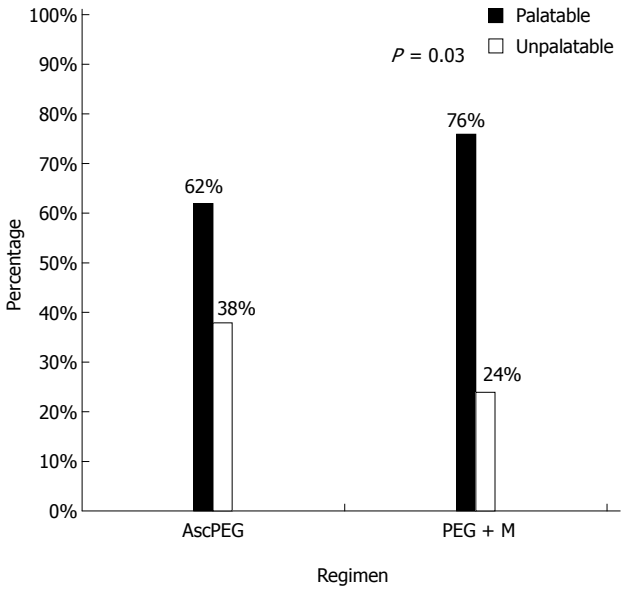

Figure 3 Palatability of the colon preparation (score ⩽ 3: Unpalatable; 4 or 5: Palatable). *P*-value for the difference in palatability between AscPEG and PEG + M is 0.03. PEG + M: PEG electrolyte solution + menthol; AscPEG: Ascorbic acid PEG electrolyte solution.

administer, palatable, well tolerated, safe and effective. Despite the unquestionable need for adequate colon cleansing, suboptimal bowel preparation occurs with surprising frequency in as many as 25% to 40% of cases[12]. Inadequate bowel preparation is associated with canceled procedures, prolonged procedure time, incomplete examination, increased cost, and missed pathology. Split-dose 4 L PEG is superior to other comparator preparations and is considered the gold standard against which other regimens should be compared[15,30]. Although highly effective even with minimal dietary restriction, split-dose 4 L PEG is limited by the high volume and unpleasant taste leading to poor acceptability by patients. To circumvent the volume and taste issue, a new formulation of PEG including ascorbic acid was developed (AscPEG). The added megadose of ascorbic acid is not completely absorbed, exerting an osmotic effect in the colon thereby reducing the necessary effective volume of cleansing solution to 2 L. When compared to split-dose 4 L PEG in a "quasi-randomized" study, AscPEG was similar in achieving adequate bowel preparation[28]. However, more excellent preparations were reported in the 4 L PEG arm (79% *vs* 52% for AscPEG, $P < 0.001$). No significant difference was found between the two preparations in terms of taste, tolerability or acceptability.

Another successful way to circumvent the aforementioned limitation of the split-dose 4 L PEG standard was the simple use of menthol candy (PEG + M) as an adjunct resulting in enhanced palatability, acceptability as well as a higher number of excellent preparations[26]. The results of this study confirm that this simple addition not only results in a high rate of adequate preparations, but also in improved palatability and acceptability (including willingness to retake in the future) over AscPEG so far considered a more patient-friendly preparation in terms of volume and taste.

The importance of this study is in showing that split-dose PEG + M is arguably the new and improved gold standard for combining efficacy (providing more excellent preparations), palatability, tolerability, and acceptability. Our study did not show a significant difference between the two preparations on the Ottawa scale perhaps due a type Ⅱ error and/or to inherent limitations of the Ottawa scale. Current bowel preparation scales have significant limitations in distinguishing effective preparations and, with the exception of the relatively simple yet highly subjective modified Aronchick scale, in providing a validated and clinically relevant separation between distinct stages of the full spectrum of bowel cleanliness. For example, the Ottawa scale is overly sensitive to the presence of residual liquid in the colon (even when that liquid is clear) leading to a final score that may not necessarily reflect visualization of the mucosa[31] and which is not readily convertible into the relevant subjective ratings generally used by endoscopists. In our study this limitation of the Ottawa scale was evident when we tried defining broad categories (adequate *vs* inadequate or excellent *vs* less than excellent) based on numerical clustering. In practice, clinicians are not interested in such complex scoring systems and prefer to rely instead on a simple subjective dichotomous scoring system: adequate *vs* inadequate. The limitations of the various bowel preparations scales and the non-inferiority design of most studies of bowel preparation are equally important considerations as small, yet important differences, may go unnoticed in clinical trials.

A recent editorial by Rex ushered in an era of increased expectations for the efficacy of bowel preparations noting that "efficacy and tolerability are related, and together constitute the main ingredients of effectiveness"[30]. The performance characteristics of colonoscopy are highly dependent on the quality of the preparation. A recent study showed that a fair bowel preparation is associated with 18-fold increase in the odds of receiving a post-colonoscopy surveillance recommendation that is inconsistent with current guidelines[32]. Narrowing the gap by shifting to safe, palatable, tolerable, and highly effective preparations is therefore a necessity that appears increasingly achievable.

Our study has few limitations. The study was conducted at a single center limiting the generalizability of the results and the sample size may have underestimated some true differences between the two interventions. Patients receiving PEG + M were provided with free packets of sugar-free menthol candy and it is unclear if, outside of clinical trials, patients will realize the value of this simple addition,

purchase the menthol candy (retail price of about $2 for a packet of 25 candies), and follow the simple instructions for use. An accurate estimate of patient compliance to dietary changes was not recorded but is likely a minimal factor given the largely unrestricted diet. Lastly, we did not examine intra or interobserver variability, however, a scoring bias is unlikely given the random study design and the blinding of the endoscopists.

In summary, this study confirms that both split-dose preparations are effective at achieving adequate colon cleansing with minimal dietary restrictions. Menthol-enhanced split-dose 4 L PEG (PEG + M) was superior to split-dose 2 L AscPEG in terms of palatability, acceptability, and excellence in quality. The simple addition of menthol candy is an easy and safe strategy and leads to improved effectiveness of split-dose 4L PEG and to a favorable quality improvement that should further enhance the power of colonoscopy.

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COMMENTS

Background

Colon preparations are poorly tolerated.

Research frontiers

Improving palatability and tolerability of bowel purgative solutions are key elements to effective bowel cleansing.

Innovations and breakthroughs

Both bowel preparations are effective. However, polyethylene glycol electrolyte (PEG) plus menthol candy is more palatable than ascorbic-acid PEG and is associated with better tolerability and high quality bowel preparation.

Applications

The simple adjuvant use of menthol candy with PEG improves tolerability and adherence.

Peer-review

The authors have performed a well conducted single blind randomized study comparing menthol-enhanced PEG *vs* PEG-ascorbic acid for colonoscopy preparation. They've carried out an important study in the realm of colonoscopy bowel preparation and have done an excellent job of conveying its importance not only in regards to the necessity of an effective bowel preparation for a good colonoscopy exam, but the role palatability has in this respect and the implications of a poor exam including inappropriate surveillance exam intervals. The study is novel in that examination of two well-tolerated and effective bowel preparations have not been examined head-to-head previously, with findings that should have a significant impact in the future administration of an effective and tolerable bowel preparation.

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Gastrointestinal neuroendocrine tumors treated with high dose octreotide-LAR: A systematic literature review

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Abstract

AIM: To review literature on efficacy and safety of octreotide-long-acting repeatable (LAR) used at doses higher than the Food and Drug Administration (FDA)-approved 30 mg/mo for treatment of neuroendocrine tumors (NETs).

METHODS: We searched PubMed and Cochrane Library from 1998-2012, 5 conferences (American Society of Clinical Oncology, Endocrine Society, European Neuroendocrine Tumor Society, European Society for Medical Oncology, North American Neuroendocrine Tumor Society) from 2000-2013 using MeSH and key-terms including neuroendocrine tumors, carcinoid tumor, carcinoma, neuroendocrine, and octreotide. Bibliographies of accepted articles were also searched. Two reviewers reviewed titles, abstracts, and full-length articles. Studies that reported data on efficacy and safety of $\geqslant$ 30 mg/mo octreotide-LAR for NETs in human subjects, published in any language were included in the review.

RESULTS: The search identified 1086 publications, of which 238 underwent full-text review (20 were translated into English); 17 were included in the review. Studies varied in designs, subjects, octreotide-LAR regimens, and definition of outcomes. Eleven studies reported use of higher doses to control symptoms and tumor progression, although symptom severity and formal quality-of-life analysis were not quantitatively measured. Ten studies reported efficacy, describing 260 subjects with doses ranging from 40 mg/mo or 30 mg/3 wk up to 120 mg/mo. Eight studies reported expert clinical opinion that supported dose escalation of octreotide-LAR up to 60 mg/mo for symptom control and suggested increased doses may be effective at preventing tumor progression. Eight studies reported safety; there was no evidence of increased toxicity associated with doses of octreotide-LAR > 30 mg/mo.

CONCLUSION: As reported in this review, octreotide-

LAR at doses > 30 mg/mo is being prescribed for symptom and tumor control in NET patients. Furthermore, expert clinical opinion provided support for escalation of somatostatin analogs for refractory hormonal symptoms.

Key words: Carcinoma; Neuroendocrine; Carcinoid syndrome; Carcinoid tumor; Gastrointestinal neoplasms; Neuroendocrine tumor; Antineoplastic agents; Hormonal; Dose-Response relationship; Drug; Octreotide; Literature review; Efficacy; Effectiveness; Symptom control; Tumor progression control; Diarrhea; Abdominal pain; Flushing

Core tip: Although octreotide-long-acting repeatable (LAR) is Food and Drug Administration approved for alleviating severe diarrhea/flushing associated with metastatic carcinoid tumors at doses ⩽ 30 mg every 4 wk, our review found that several studies within the published literature described the use of above-label doses of octreotide-LAR for symptom and tumor control of neuroendocrine tumors. Expert clinical opinion, as reported in this review, supports escalation of somatostatin analogs for patients with refractory hormonal symptoms.

Broder MS, Beenhouwer D, Strosberg JR, Neary MP, Cherepanov D. Gastrointestinal neuroendocrine tumors treated with high dose octreotide-LAR: A systematic literature review. *World J Gastroenterol* 2015; 21(6): 1945-1955 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i6/1945.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i6.1945

INTRODUCTION

Gastrointestinal neuroendocrine tumors (NETs) are rare and generally slow-growing neoplasms that secrete various peptides and neuroamines, some of which cause clinical symptoms[1]. The annual incidence of NETs is approximately 40-50 cases per million, which is about 1%-2% of all gastrointestinal malignancies. These tumors may be classified in multiple ways but are often divided into carcinoid tumors and pancreatic neuroendocrine tumors (pNET). Symptoms are often nonspecific, leading in many cases to delayed diagnosis.

First-line systemic therapy for NETs often consists of somatostatin analogs (SSAs) such as octreotide acetate (Sandostatin®; Novartis Pharmaceutical Company, East Hanover, NJ, United States) or lanreotide (Somatuline®; Ipsen Pharmaceuticals, Paris, France). These drugs, initially developed to palliate the symptoms of carcinoid syndrome, have an inhibitory effect on secretion of gastrointestinal hormones (*e.g.*, serotonin). Accumulating data indicate that SSAs are also capable of inhibiting NET growth[2,3]. Currently, the maximum FDA-approved dosage and administration of octreotide long-acting repeatable (LAR), indicated for severe diarrhea/flushing episodes associated with metastatic carcinoid tumors and VIPomas, is 30 mg every 4 wk[4].

A recent physician expert consensus panel highlighted the appropriateness of using standard dose SSAs for control of hormonal symptoms and tumor growth in patients with advanced carcinoid tumors, as well as increasing dose/frequency of SSAs in treatment of refractory carcinoid syndrome[2]. The panel also recommended that increase in the dose/frequency of SSAs be considered for patients with radiographic progression, particularly in cases where disease was previously stabilized at a lower dose. Additionally, in clinical practice, octreotide-LAR is sometimes prescribed at above-label doses but evidence for this practice has not been systematically assessed. Motivated by the recent expert panel consensus and anecdotal evidence from clinical practices, our aim was to systematically review the published literature on efficacy and safety of octreotide-LAR used at doses higher than the Food and Drug Administration (FDA)-approved 30 mg and/or administered at a frequency greater than every 4 wk for management of NETs.

MATERIALS AND METHODS

Data sources and search strategy

We searched PubMed and the Cochrane Library databases and five conferences: American Society of Clinical Oncology (ASCO) annual meeting and ASCO Gastrointestinal Cancers Symposium, Endocrine Society (ENDO), European Neuroendocrine Tumor Society (ENETS), European Society for Medical Oncology (ESMO), and North American Neuroendocrine Tumor Society (NANETS). In supplementing our search, we also reviewed reference lists of records included for data abstraction and asked for article nominations from physician experts that were part of a NET treatment consensus panel[2].

We searched PubMed using the keywords and Medical Subject Headings terms: "neuroendocrine tumors", "carcinoid tumor", and "carcinoma, neuroendocrine", and "octreotide". Our search was limited to human subjects and studies published from 1998 to present. The search year limit of "1998" was imposed based on octreotide acetate LAR (Sandostatin LAR) original approval date (www.accessdata.fda.gov/). In addition to the terms used in the PubMed search, the Cochrane Library was also searched using word combinations of "octreotide" and limited to Cochrane Reviews and Protocols. Conference abstracts were searched using an expanded version of search terms listed above: neuroendocrine tumor, neuroendocrine tumour(s), neuroendocrine carcinoma(s), carcinoid tumor(s), carcinoid tumour(s), carcinoid syndrome, octreotide, and sandostatin. The conference search was limited to studies in human subjects and published in 2000 or later.

Screening and selection criteria

Identified articles were screened in three phases, article title and abstract review, first full article review, and second full article review, and conference abstracts in one phase. Articles were advanced to the next phase of screening only if they met the screening criteria or if it was unclear if they met the screening criteria in the prior screening phase. The first and second screening phases were done by a single reviewer and the third by two reviewers. In the first phase, titles and abstracts of identified studies were screened for data in human subjects on the drug (octreotide-LAR) and condition (neuroendocrine tumors) of interest. If an article that passed this screening phase was not in English, it was translated and moved to phase two (first full article review). In phase two, studies were screened for use of octreotide acetate LAR at doses > 30 mg/4 wk. In phase three, an article was excluded if an outcome of interest (efficacy, safety, and/or expert clinical opinion) was not reported or if the article was a case report, since case reports present data on too few patients to be able to draw any generalizable conclusions. Conference abstracts were screened by a single reviewer in one phase. Studies that passed the screening were abstracted. All screening and abstraction of the identified studies was done using specialized software[5].

Outcome measures

The outcomes of interest included reports of efficacy/effectiveness and safety surrounding the use of octreotide-LAR at doses higher than 30 mg or administered at a frequency greater than 4 wk. We also reviewed expert clinical opinion statements surrounding the use of above-label use of octreotide-LAR. Measures of efficacy included symptom burden, disease markers, tumor response, time to progression of disease, requirements for additional intervention, and survival. Measures of safety included frequency of various adverse effects.

Quality assurance

The weighted Kappa inter-rater reliability was calculated between the two reviewers at the screening phase using an independently-screened sample of articles, considering that a Kappa value ranging from 0.81 to 0.99 is interpreted to indicate "almost perfect agreement"[6]. After determining that the inter-rater reliability was high (*i.e.*, > 0.81)[6], the remaining articles were divided between the two reviewers. Data were also first independently extracted from a sample of accepted articles by the two reviewers, and any resultant inconsistencies were discussed. The remaining articles were then split up between the two reviewers to finalize data extraction. Further accuracy of data abstraction was assured by ongoing consultation between the reviewers and the research team, such that ambiguities were resolved *via* consensus. The Oxford's Centre for Evidence-based Medicine Levels of Evidence (OCEBM) was used to assign a quality of evidence grade of 1 to 5 to each included study, with 1 indicated a study with the strongest scientific basis for support of conclusions and 5 the weakest (*e.g.*, expert opinion)[7].

RESULTS

Search and screening

The PubMed and the Cochrane Library databases searches, conducted on 12/9/12, yielded 705 articles: 692 were identified in PubMed, 13 in the Cochrane Library database, and 3 more provided by experts (Figure 1). A search of conferences resulted in 116 abstracts identified from the ASCO meetings (87 from 2000-2012 annual meetings; 29 from 2004-2013 Gastrointestinal Cancers Symposiums), 83 from 2000-2012 ENDO meetings, 92 from 2004, 2006, 2009-2012 ENETS meetings, 29 from 2000 and 2002-2012 ESMO meetings, and 54 from 2008-2012 NANETS meetings. An update to the search was conducted in June 2013 to identify articles in the ASCO 2013 meeting, which resulted in 4 more abstracts. A total of 708 articles and 378 conference abstracts were screened.

In the first phase of screening, 848 of 1086 records were excluded from further screening (Figure 1). Twenty articles were translated before phase two (2 from Spanish, 6 from Italian, 4 from Japanese, 1 from French, 6 from Hungarian, and 1 from Danish). In phase two, 205 of 230 full-length articles were excluded due to lack of data on the use of octreotide acetate LAR at doses > 30 mg/mo. In the final phase, 15 of 33 additional articles (10 did not discuss outcomes of interest and 5 were case reports) were excluded. Bibliographies of the 10 accepted full-length articles were screened for additional studies of interest but no additional studies were identified. Eight of the 378 identified abstracts passed the screening and were abstracted. The weighted kappa for the two reviewers was 0.94[6]. During abstraction, it was determined that 2 of the 10 included articles reported data on the same patients, hence only the most relevant and recent of the two studies (Woltering 2006), which reported data on outcomes of interest, was retained in this review.

Description of included studies

The review consisted of 17 studies, including 8 conference abstracts and 9 articles (Table 1). Four were clinical studies, one was a report of a modified Delphi process[2], one was a systematic review[21], two were non-systematic reviews[8,9] and one was a letter to the editor[10]. Seven conference abstracts reported on clinical studies and one a randomized phase Ⅲ trial. Eight studies were performed in the United States, two in Italy, one in both the United States and Europe, and the location for the remaining 6 was not reported.

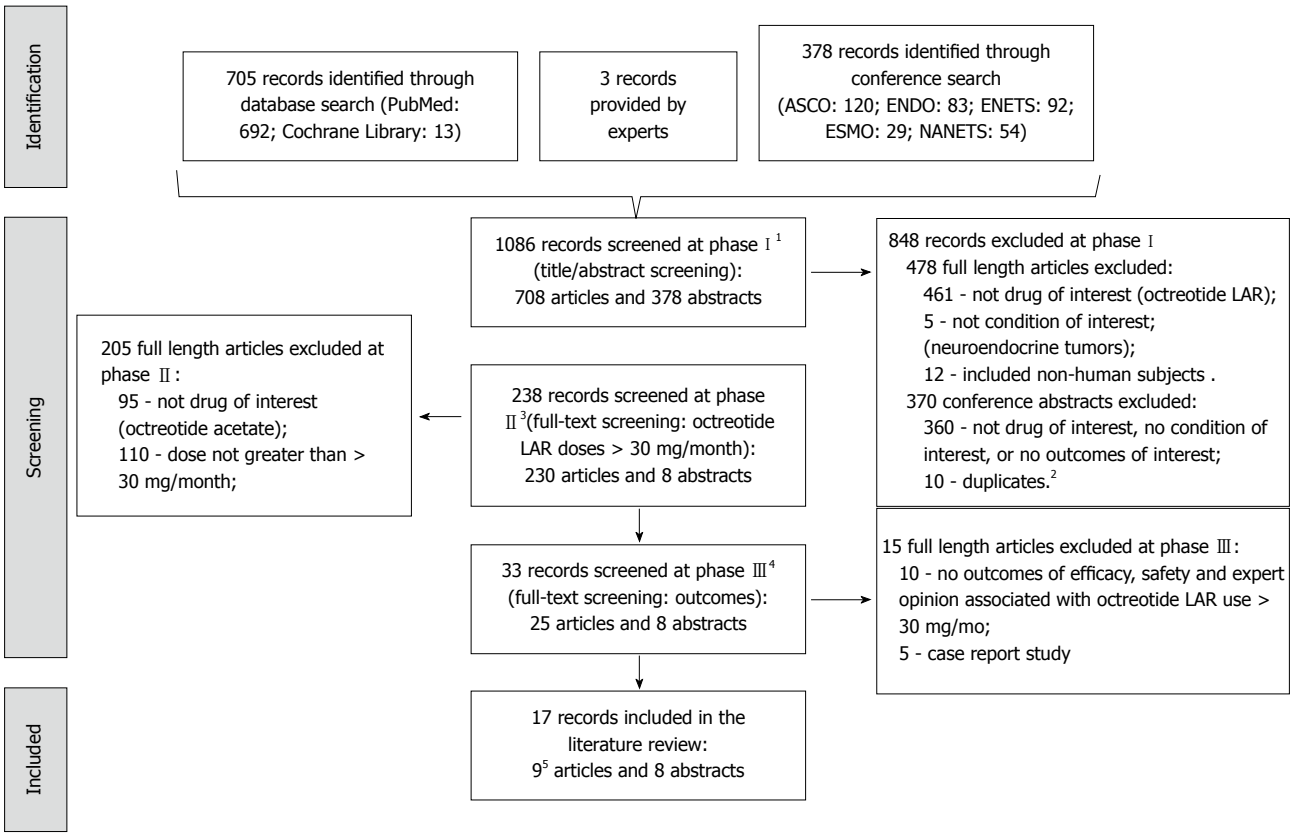

Figure 1 Record search and screening flow chart. [1]Phase Ⅰ: Title and abstract screen of all identified records through database search; screens for records that contain: octreotide LAR, neuroendocrine tumors, and human subjects. [2]Duplicates include duplicate abstracts presented at different conferences and data reported in full-length articles included in this review. [3]Phase Ⅱ: Full-text screen of those records that passed phase Ⅰ; screens for records containing use of octreotide LAR of doses greater than 30 mg/4 wk. [4]Phase Ⅲ: Full-text screen of those records that passed phase Ⅱ; screen for records containing safety and effectiveness outcomes, and exclude records that are case reports. [5]Two of the 10 included articles reported data on the same patients, hence only the most recent study (Woltering 2006) was retained in this review. American Society of Clinical Oncology (ASCO), Endocrine Society (ENDO), European Neuroendocrine Tumor Society (ENETS), European Society for Medical Oncology (ESMO), and North American Neuroendocrine Tumor Society (NANETS).

Ten studies considered efficacy, 8 addressed safety, and eight provided expert clinical opinion. OCEBM grades ranged from 2b to 5, with 9 studies out of 17 scoring 4 or 5.

Efficacy

The efficacy of high dose octreotide-LAR was reported in 10 studies, of which 4 were full-length articles[11-20] (Table 2). Doses studied ranged from a minimum of either 40 mg per month or 30 mg per 3 wk up to a maximum of 120 mg per month. The studies describe over 260 subjects who received increased doses of octreotide-LAR (the exact number cannot be stated with certainty, as some studies did not report the number of patients treated with above-label doses and grouped them with other patients).

Chadha *et al*[12] retrospectively studied high dose octreotide-LAR used to treat 30 patients with GEP-NETs. Compared to 24 subjects receiving conventional dose octreotide-LAR therapy, there was a trend (P = 0.12) toward increased time before further treatment intervention was required in this group but no effect on survival (P = 0.48). Ferolla[14] was the only prospective trial of high dose octreotide-LAR, examining the effect of 30 mg every 3 wk on tumor progression, serum markers and symptoms. The comparator group was historical and consisted of the same subjects receiving octreotide-LAR 30 mg/mo. Control of symptoms was seen with increased dose of octreotide-LAR, with 40% noting normalization and 60% noting control of symptoms (flushing, diarrhea, and bronchospasm). The time to tumor progression and the time to biochemical progression (increase in serum CGA, serum gastrin and/or urinary 5-HIAA) were both significantly delayed (P < 0.01). The study reported that "weakness and well-being improved in all patients" but a specific tool for measuring quality of life was not described. One of the larger studies[11] reported physician practice patterns in medical management of 392 patients with NETs. Data were reported by doses delivered rather than by patients treated, so it was not possible to compare this study to the other studies of efficacy that used more standard methodology. Tumor responses to doses of octreotide-LAR 20 and 30 mg were similar to those at doses of 40 and 60 mg. Woltering[20] examined whether CgA levels correlated with octreotide-LAR dose and symptoms in 40 patients with carcinoid

Table 1 Description of included studies

Ref. (OCEBM)	Country, timeframe	Study design	Study subjects	Doses of SLAR administered (number of patients or number of doses)	Reasons for high dose SLAR[2]	Relevant outcomes reported
Strosberg *et al*[2] 2013 (5)	United States	Modified delphi process	404 patient scenarios of patients with unresectable metastatic well-differentiated carcinoid tumors	Subset of patient scenarios treated with SLAR (frequency: every 2 wk, 3 wk, 4 wk; dosing 30 mg, 40 mg, 60 mg, 90 mg, 120 mg)	NA/NR	Expert clinical opinion
Colao *et al*[8] 2010 (5)	Italy	Literature review	Pituitary tumors and NETs	NA/NR	NA/NR	Expert clinical opinion
Oberg *et al*[9] 2004 (5)	Europe, United States	Literature review	Relating to pts. with GEPNETs	NA/NR	NA/NR	Expert clinical opinion
Yao *et al*[10] 2008 (5)	United States	Letter to editor	Relating to pts. with NETs	NA/NR	NA/NR	Expert clinical opinion
Anthony *et al*[11] 2011 (3b)	United States, 2000-2006	Retrospective multicenter medical chart review	392 pts. with functioning NETs (with or without CS), treated with SLAR ⩾ 4 mo	10 mg (22 doses), 20 mg (224), 30 mg (316), 40 mg (78), 50 mg (16), 60 mg (42)	Lack of efficacy	Efficacy, safety
Chadha *et al*[12] 2009 (2b)	United States, 2002-2007	Retrospective single-center medical chart review	54 pts. with GEPNETs treated with SLAR 20-30 mg	Median high dose of SLAR 40 mg, ranging 40-90 mg/mo (30 pts.)	Control of symptoms, treatment of tumor	Efficacy, safety, Expert clinical opinion
Costa *et al*[13] 2006[1] (4)	2005	Retrospective case series	9 pts. with progressive metastatic NETs treated with SLAR 20 mg/mo	20 mg/mo (9 pts.); 30 mg/mo, 40 mg/mo (3 pts.)	Treatment of tumor	Efficacy, Expert clinical opinion
Ferolla *et al*[14] 2012 (2b)	Italy	Multicenter open-label non-randomized prospective clinical trial	28 pts. with well-differentiated NETs, progressing at standard dose SLAR ⩾ 6 mo	30 mg/28 d initially for 6-32 mo (28 pts.); 30 mg/21 d (28 pts.) for 4-48 mo	Control of symptoms, treatment of tumor, other	Efficacy, safety
Koumarianou *et al*[15] 2010[1](4)	2008-2009	Retrospective case series	13 pts. with pretreated progressive metastatic GEPNETs	30 mg/3 wk (12 pts.)	Treatment of tumor	Efficacy, safety
Markovich *et al*[16] 2012[1] (4)		Retrospective clinical trial	31 pts. with pretreated progressing disseminated NETs	20 mg/mo initially (29 pts.); 30 mg/mo (18); 40 mg/mo (13) long acting octreotide	Control of symptoms, treatment of tumor, other	Efficacy, safety
Valle *et al*[17] 2001[1] (4)		Retrospective single-center case series	8 pts. with metastatic carcinoid syndrome	Initially 20 mg/mo (5 pts), 15 mg/mo, 60/mo, 20/2 wk (all 1); escalated to 20 mg/3 wk, 30 mg/3 wk, 50 mg/4 wk, 120 mg/4 wk (4 pts.)	Control of symptoms	Efficacy, safety
Weber *et al*[18] 2012[1] (4)	United States, 2000-2010	Retrospective single-center medical chart review	337 pts. with metastatic small-bowel carcinoid tumors, treated with SLAR	27% (99 pts.) with increased high dose; common max doses were 40 mg/4 wk (37 pts.), 60 mg/mo (34), 30 mg/3 wk (17)	Control of symptoms, tumor progression, other	Efficacy
Wolin *et al*[19] 2013[1] (2b)		Multicenter randomized phase Ⅲ clinical trial	110 pts. with unresponsive NET symptoms to standard dose SLAR	40 mg/28 d (57 pts.)	Control of symptoms	Efficacy, safety
Woltering *et al*[20] 2006 (3)	United States	Non-randomized prospective clinical trial	40 pts. with carcinoid syndrome on stable doses of SLAR for ⩾ 3 mo	20 mg (8 pts.), 30 mg (19), 60 mg (13) SLAR/mo	Control of symptoms	Efficacy
Ludlam *et al*[21] 2011 (3a)	1965-2010	Systematic literature review	Relating to pts. with NETs	NA/NR	Control of symptoms	Safety
Strosberg *et al*[22] 2013[1] (2b)	United States, 2004-2010	Retrospective multicenter medical chart review	271 pts. with carcinoid and pancreatic NETs, treated with octreotide-LAR	40% (n = 82) of carcinoid pts had high dose: common max doses of 40 mg/mo (39%), 40 mg/3 wk (18%), 30 mg/2 wk (17%); and 23% (15) of pNET pts: 40 mg/mo (33%), 30 mg/2 wk (27%), 60 mg/mo (27%)	Control of symptoms, treatment of tumor, other	Expert clinical opinion

Xu *et al*[23] 2012[1] (3b)	United States, 1999-2007	Retrospective analysis of SEER-Medicare claims	355 pts. with NETs	26 pts. (7.3%) with ≤ 10 mg initially, of which 3.9% increased to > 40 mg; 91 (25.6%) with 11-20 mg initially, 5.5% increased to 31-40 mg and 4.4% to > 40 mg; 147 (41.4%) with 21-30 mg initially, 11.6% increased to 31-40 mg and 10.9% to > 40 mg; 65 (18.3%) with 31-40 mg initially, 86.2% increased to 31-40 mg and 13.9% to > 40 mg; 26 (7.3%) with > 40 mg initially, 100% increased to > 40 mg; 134 pts. (37.7%) escalated to > 30 mg/mo during 1st year of therapy	NA/NR	Expert clinical opinion

[1]Conference abstract; [2]Control of symptoms (flushing, diarrhea, and *etc.*); treatment of tumor (tumor progression); other (biochemical markers). NA/NR: Not applicable/not reported; OCEBM: Oxford's Centre for Evidence-Based Medicine Levels Of Evidence; CS: Carcinoid syndrome; NETs: Neuroendocrine tumors; GEPNETs: Gastroenteropancreatic neuroendocrine tumors; SLAR: Octreotide long-acting repeatable; pts.: Patients.

syndrome, and determined that CgA levels were similar for those receiving octreotide-LAR 20 mg, 30 mg or 60 mg/mo.

Six conference abstracts reported efficacy of high dose octreotide-LAR[13,15-19]. Costa[13] analyzed characteristics and clinical outcomes in patients with progressive metastatic NETs treated with octreotide-LAR. The authors noted that "after evidence of objective progressive disease in liver, a second response with disease stabilization was achieved with octreotide dosage increase from 30 mg to 40 mg every 4 wk" in two subjects that received high dose octreotide-LAR. Koumarianou[15] studied efficacy of octreotide-LAR (30 mg/3 wk) combination therapy in 13 subjects with NETs. There was no comparator group. Disease remained stable in 25% (3/12) and 75% showed over > 50% reduction in tumor size. The median progression free survival in evaluable patients was 24.6 wk. Markovich[16] studied the efficacy of long acting octreotide (including octreotide-LAR, Octreotide-Depot and Octreotide-LONG) 30-40 mg/mo in heavily pretreated patients with disseminated NETs. Subjects (n = 29) initially received long-acting octreotide 20 mg/mo and the dose was escalated to 30 mg/mo (n = 18) or 40 mg/mo (n = 13). The reason for dose escalation was disease progression in 19 and lack of symptom control in 12. Those receiving long-acting octreotide alone (n = 14) did not have a response. However, tumor growth was controlled in 25 subjects (80.7%). Median time to progression in the total group was 18 mo. The report did not indicate what percentage of subjects specifically received octreotide-LAR *vs* other forms of long acting octreotide. Valle *et al*[17] determined patient acceptability and control of carcinoid-related symptoms in 8 patients receiving octreotide-LAR, two of which started on high dose. Three subjects required dose escalation to high dose octreotide-LAR to control symptoms. Valle *et al*[17] reported "patients who required continued dose-escalation of Sandostatin LAR showed evidence of increased urinary 5-HIAA suggesting increased disease activity." Weber *et al*[18] determined the frequency of "above-label" octreotide-LAR dosing and outcomes. Of 337 subjects treated with octreotide-LAR for metastatic NETs at a single tertiary care institution, 99 (27%) received high dose. The indications for dose increase were worsening carcinoid syndrome (60 patients), radiographic progression (33 patients), and rising urine 5-HIAA (6 patients). Among patients whose doses were increased for refractory carcinoid syndrome, flushing improved in 56% and diarrhea improved in 62%. Finally, a randomized phase Ⅲ clinical trial by Wolin *et al*[19] showed that treatment with octreotide-LAR to 40 mg/4 wk led to better control of diarrhea and flushing at 6 mo after starting treatment. Progression free survival was 6.8 mo. The comparator arm in this trial was a novel long-acting somatostatin analog (pasireotide-LAR 60 mg/4 wk), which showed a longer progression free survival (11.8 mo).

Considering these studies together, there is a trend supporting the use of octreotide-LAR at above-label doses to control symptoms and tumor progression.

Safety

The safety of high dose octreotide-LAR was reported in 8 studies[11,12,14-17,19,21] (Table 3). There was no increased toxicity compared to conventional dose therapy reported in 3 of the studies that examined safety[12,17,21]. Wolin *et al*[19] compared octreotide-LAR treatment arm to patients using pasireotide-LAR, and showed the two arms have a similar safety profile (diarrhea: 7% *vs* 9%; abdominal pain: 9% *vs* 2%) except for the higher frequency of hyperglycemia (0% *vs* 11%) in the pasireotide group, and 4 (7%) and 7 (13%) patients that discontinued due to adverse events. Overall, our review shows that adverse events were not well described. This may be because octreotide-LAR has a favorable safety profile and that modest increases in the dose may not lead to significant

Table 2 Efficacy of octreotide long-acting repeatable > 30 mg/mo

Ref.	Symptoms	Disease markers[2]	Tumor response/Disease progression
Anthony *et al*[11] 2011	NA/NR	NA/NR	Complete response rates: 2% (20 mg), 1% (30 mg), 0% (40 mg), 2% (60 mg); partial response: 6% (20mg), 8% (30 mg), 4% (40 mg), 10% (60 mg); stable disease: 57% (20 mg), 57% (30 mg), 55% (40 mg), 50% (60 mg); progression: 21% (20 mg), 25% (30 mg), 18% (40 mg), 29% (60 mg)
Chadha *et al*[12] 2009	NA/NR	NA/NR	Median time to intervention was 2.9 mo (conventional dose group) *vs* 17.7 mo (high-dose) (P = 0.12)
Costa *et al*[13] 2006[1]	NA/NR	NA/NR	After evidence of progressive disease in liver, disease stabilization was achieved with increase to 30 mg and to 40 mg/mo
Ferolla *et al*[14] 2012	Complete normalization: 40%; partial symptom control: 60%; flushing (normalized in 71.4%, improved in 28.6%), diarrhea (70%, 30%); pain (disappeared in 33%; improved in 67%); bronchospasm improved in 100%; hypoglycemia improved in 100%; weakness/well-being improved in all pts	30% of pts. with elevated markers responded to higher dose; significant response to high dose SLAR was in 30% of pts. with high CgA, 57.1% of pts. with high 5-HIAA, and 100% of pts. with high gastrin; median time-to-biochemical progression was 30 mo (SLAR 30 mg/21 d) *vs* 14 mo (standard dose) (P < 0.01)	Partial response in 7.2%, stable disease in 92.8%; median time to progression was 30 mo. (SLAR 30 mg/21 d) *vs* 9 mo. (standard dose) (P < 0.0001);
Koumarianou *et al*[15] 2010[1]	NA/NR	NA/NR	75% (9/12) with > 50% tumor size reduction, and 25% had stable disease; median PFS was 24.6 wk
Markovich *et al*[16] 2012[1]	NA/NR	NA/NR	Partial response in 3.2%, stable disease in 80.7%, progressed in 16.1%; tumor growth control in 83.9%, pts. had biochemical response and symptom relief (results not broken down by dose)
Valle *et al*[17] 2001[1]	Improvement in flushing, diarrhea, and bronchospasm (results not broken down by dose)	pts. with dose-escalation of SLAR had increased 5-HIAA, suggesting increased disease activity	
Weber *et al*[18] 2012[1]	In pts., with increased doses of SLAR for refractory CS, 62% had improvement in diarrhea and 56% had improvement in flushing	NA/NR	NA/NR
Wolin *et al*[19] 2013[1]	At month 6, symptom response was 21% (9/43) in pasireotide-LAR *vs* 27% (12/45) in ocrteotide-LAR arms (OR = 0.73; 95%CI: 0.27-1.97; P = 0.53); 36.5% ± 29.1% had a reduction in diarrhea/d and a 49.4% ± 36.7% in flushing/2 wk	NA/NR	Median PFS was 6.8 mo in octreotide-LAR *vs* 11.8 mo pasireotide-LAR arms (HR = 0.46; P = 0.045)
Woltering *et al*[20] 2006	Flushing not controlled in 0% (20 mg), 11.1% (30 mg), *vs* 7.1% (60 mg) SLAR groups (P = 1.0); diarrhea not controlled in 0% of pts. (20 mg), 27.8% (30 mg), *vs* 30.8% (60 mg) groups (P = 0.3182)	Mean absolute serum CgA: 53.1 (20 mg SLAR), 65.8 (30 mg), 70.7 (60 mg) (P = 0.9847); mean absolute plasma CgA: 56.6 (20 mg), 66.2 (30 mg), 65.2 (60 mg) (P = 0.9092)	NA/NR

[1]Conference abstract; [2]Disease makers: 5-hydroxyindoleacetic acid (5-HIAA), chromogranin A (CgA), gastrin, and insulin. NA/NR: Not applicable/not reported; CS: Carcinoid syndrome; SLAR: Octreotide long-acting repeatable; PFS: Progression free survival.

increases in toxicity, or because the studies were too small to identify rare events.

Expert clinical opinion

Expert clinical opinions were reported in 8 articles[2,8-10,12,13,22,23] (Table 4). Both Oberg *et al*[9] and Strosberg *et al*[2] supported the idea of increasing octreotide-LAR dose up to 60 mg/mo for control of symptoms. Chadha *et al*[12], Colao *et al*[8], and Costa *et al*[13] further suggested that increased doses of octreotide-LAR may be effective for tumor progression. Strosberg *et al*[22] reported above-label dosing of octreotide-LAR is common in NCCN institutions, and the primary indication is refractory carcinoid syndrome. Yao *et al*[10] cautioned that until there are appropriate studies (*i.e.*, randomized controlled trials) completed, escalating doses of octreotide-LAR should be done only for control of symptoms. Xu *et al*[23]

Table 3 Safety of octreotide long-acting repeatable > 30 mg/mo

Ref.	Safety
Anthony *et al*[11] 2011	NA/NR (adverse events not broken down by SLAR dose); of 392 pts., 8.7% had hyperglycemia, 6.4% had cholelithiasis, 2.8% had cholecystitis, 2.3% had steatorrhea, and 1.5% had hypoglycemia, and 22% had ≥ 1 adverse event during SLAR use
Chadha *et al*[12] 2009	p 4129: "No treatment related toxicities were reported."
Ferolla *et al*[14] 2012	p 329: "No additional toxicity was recorded for the schedule treatment with LAR 30 mg every 21 d when compared with standard LAR dose and no treatment discontinuation or dose modification was required. Adverse events observed in patients in treatment with LAR 30 mg every 21 d were diarrhea in 1, abdominal pain in 1, cholelithiasis in 2, pyrexia in 1 patient. No constipation, dizziness, arterial hypertension or any other adverse event was observed."
Koumarianou *et al*[15] 2010[1]	"Patients reported no significant symptoms related to treatment adverse events. Two patients experienced a grade Ⅰ neutropenia and one patient a grade Ⅱ thrombocytopenia. One patient did not receive treatment due to persistent nausea and vomiting resistant to metoclopramide."
Ludlam *et al*[21] 2011	p 838: "A trial designed to compare two dose levels of octreotide LAR (30 and 40 mg/mo) highlighted the ability of octreotide LAR to control diarrhea in patients with active or prior chemotherapy-induced diarrhea. Fewer patients in the 40 mg/mo group compared with those in the 30 mg/mo group experienced severe diarrhea (62% *vs* 48%; $P = 0.14$), required intravenous fluid (32% *vs* 19%; $P = 0.10$), or had diarrhea-related unscheduled healthcare visits (42% *vs* 28%; $P = 0.11$). Most importantly, adverse events were balanced between the two groups."
Markovich *et al*[16] 2012[1]	"Tolerability of long-acting octreotide in a dose of 30-40 mg was satisfactory for all pts."
Valle *et al*[17] 2001[1]	"All patients found the long-acting analogue acceptable and none requested a change back to conventional daily octreotide. Sandostatin LAR is an alternative somatostatin analogue that is highly acceptable to patients; doses may be safely escalated if required."
Wolin *et al*[19] 2013[1]	"Hyperglycemia (11% *vs* 0%), diarrhea (9% *vs* 7%), and abdominal pain (2% *vs* 9%) were the most common grade 3/4 AEs in the pasireotide-LAR (P) *vs* octreotide-LAR (O) arms in the core phase, and 7 (13%) and 4 (7%) patients discontinued due to AE. P and O showed a similar safety profile except for the higher frequency of hyperglycemia in P."

[1]Conference abstract. NA/NR: Not applicable/not reported.

Table 4 Expert clinical opinion statements on use of octreotide long-acting repeatable > 30 mg/mo

Ref.	Quoted expert clinical opinion
Chadha *et al*[12] 2009	p 4130: "Our experience and those of others have shown that S-LAR can provide disease control to prolong time needed for liver-directed therapies and systemic therapies. Dose escalation provides an opportunity to spare patients from morbidities associated with these procedures and systemic therapy."
Colao *et al*[8] 2010	p 290: "The dosages of SSAs currently used are probably insufficient to determine control of hormone secretion and tumor growth both in acromegaly and NETs."
Costa *et al*[13] 2006[1]	"Dose increase with octreotide LAR should be offered to those patients who progress after achieving a first objective response with SS analogues."
Oberg *et al*[9] 2004	p 970: "As a general rule, if the total IR dose is 200-600 μg/d, LAR 20 mg should be tried, and if total IR dose is 750-1500 μg/d, LAR 30 mg should be tried. The LAR doses range from 20 to 60 mg/28 d. Supplementary administration with the IR form of octreotide in patients escaping anti-secretory response is often required during long-term treatment with LAR. If it is necessary to give the patient rescue doses of IR octreotide three or four times per week, increase the LAR dose to 30 mg/4 wk, or reduce the interval between administrations of the depot formulation (*e.g.*, 20 mg/3 wk). Furthermore, the temporal occurrence of hypersecretion during the 4-week dosing interval should be considered. For example, if the rescue s.c. therapy is required during the week before the next injection of LAR, then a reduction of the dosing interval by 1 wk is advisable. On the other hand, if the need for rescue medication occurs sporadically throughout the month then increasing the dose stepwise by 10 mg/mo up to 60 mg/mo should be tried. Doses of LAR > 60 mg/mo are rarely of added value."
Strosberg *et al*[2] 2013	p 5: "In patients with uncontrolled secretory symptoms, increasing the dose/frequency of SSAs is appropriate (median rating, 8), particularly among patients who had previously responded to a lower dose. The panel considered dose escalations of octreotide LAR up to 60 mg/4 wk (median rating, 7) or up to 40 mg/3 wk (median rating, 7) to be reasonable adjustments for refractory carcinoid syndrome. Increasing the dose/frequency of SSAs may be considered in patients with radiographic progression, particularly those whose disease was previously stabilized at a lower dose. The panel considered an increase in dose/frequency up to 40 mg/3 or 4 wk to be reasonable (median rating: 4-5.5). There is a lack of evidence that increasing the dose/frequency of SSAs slows radiographic progression."
Strosberg *et al*[22] 2013[1]	"Above label dosing of octreotide LAR is common in NCCN institutions. The primary indication is refractory carcinoid syndrome."
Xu *et al*[23] 2012[1]	"Our analyses showed that patients frequently required the escalation of octreotide LAR dose during their course of illness. A substantial number of patients required doses greater than the FDA approved dose of 30 mg/mo."
Yao *et al*[10] 2008	p 338: "Although octreotide has proven to be a safe and efficacious drug for carcinoid syndrome, it nonetheless can cause adverse events including steatorrhea, cholelithiasis, and hyperglycemia. Until conclusive data from randomized studies are available to show that octreotide has a disease-stabilizing effect and at what does this effect occurs in humans, we advocate titrating the octreotide LAR dose according to symptoms rather than to an arbitrary blood level."

[1]Conference abstract. NCCN: National Comprehensive Cancer Network; NETs: Neuroendocrine tumors; S-LAR: Octreotide long-acting repeatable; FDA: Food and Drug Administration; SSAs: Somatostatin analogues.

reported that a substantial number of patients in this study required doses greater than the FDA approved dose of 30 mg/mo. Overall, our review indicated that clinical experts support dose escalation of octreotide-LAR.

DISCUSSION

There is a general trend supporting the use of high dose octreotide-LAR for control of symptoms and limited data supporting the use of high-dose octreotide for control of tumor progression in patients with NETs. There were no published data identified suggesting increased toxicity of octreotide-LAR at above FDA-approved dosage and frequency of administration. The lack of data may imply that there is no significant toxicity, which is consistent with reports of higher doses used in acromegaly without significant toxicity[21], or that the studies were too small to identify uncommon adverse events. Several studies provided expert clinical opinion statements, mostly endorsing use of above-label doses of octreotide-LAR for patients with symptom or tumor progression.

There were 4 articles and 6 abstracts that reported on the efficacy of octreotide-LAR > 30 mg/mo in over 260 patients[11-20]. Ferolla *et al*[14] showed efficacy of octreotide-LAR > 30 mg/mo, indicating that biochemical and tumor progression were more rapid with lower dose using historical controls. The key results reported by Weber *et al*[18], demonstrating that octreotide-LAR is commonly prescribed in doses or schedules above the label-recommended dose and frequency for refractory carcinoid syndrome, are now available in greater detail in a full-length article by Strosberg *et al*[24]. No studies showed a negative effect on efficacy of high dose octreotide-LAR. In most studies, higher octreotide-LAR doses were used as rescue or salvage therapy, and it may be that response rates would be higher in treatment-naïve patients.

Our review of 4 articles and 4 abstracts, evaluating over 220 patients[11,12,14-17,19,21], found no evidence of increased toxicity associated with doses of octreotide-LAR > 30 mg/mo. None of the studies included in this review reported significant toxicity, although these studies did not report power analyses or a priori calculations of sample size.

Expert clinical opinion expressed in 6 of 8 articles[2,8,9,12,13,22] supported the use of higher doses of octreotide-LAR when lower doses are inadequate to control disease. One study reported that above-label octreotide-LAR is commonly prescribed in NCCN institutions[22]. Most experts suggested that higher doses should be used in cases where there is tumor progression or lack of symptom control on lower doses. Yao *et al*[10] questioned the use of higher doses due to lack of high-level evidence supporting this practice.

Strengths and limitations

The strength of this review lies in its comprehensive search, review, and synthesis of global literature on above the FDA-approved label dosage and frequency of administration of octreotide-LAR in patients with NETs. This study also has limitations. The data on symptom improvement are limited by lack of quantitative measurements of symptom severity and absence of formal quality of life analysis. Nine of the 17 included studies were retrospective, and retrospective studies may be subject to recall and reporting biases. The only prospective trial reporting efficacy data in a full-length article[14] used historical controls, which may limit estimation of effects. Wolin *et al*[19] reported on a randomized clinical trial of pasireotide-LAR (comparator arm) *vs* octreotide-LAR 40 mg/28 d. However, this trial did not assess other doses of octreotide-LAR. The studies included in this review varied in design, patient population, octreotide-LAR regimens, and definitions of outcomes, and the data were reported in several ways across the reviewed studies. Thus, the heterogeneity of these data made it difficult to compare directly between studies and prevented us from conducting a meta-analysis. OCEBM grades ranged from 2b to 5, with 9 studies out of 17 scoring 4 or 5, indicating a relatively low quality of evidence (grade C)[7]. Of the 10 studies that reported data on efficacy and of the 8 that reported data on safety, 5 and 4 were small (< 100 patients) retrospective studies, respectively. Finally, in this study, we were unable to assess separately the effects of octreotide-LAR co-therapy *vs* monotherapy on safety and efficacy outcomes given the limited data for co-therapy in this review.

Future research efforts should focus on establishing efficacy and safety of prescribing octreotide-LAR at doses higher than the FDA-approved 30 mg/mo for management of neuroendocrine tumors using generalizable randomized controlled trial study designs. Studies comparing the safety of octreotide-LAR in patients treated with doses < 30 mg/mo *vs* those treated with above-label regimens of octreotide-LAR are also warranted.

In conclusion, this systematic literature review suggests that above-label doses of octreotide-LAR are being used frequently for the management of NETs in clinical practice and excess toxicity has not been observed in the reviewed studies. In most articles reviewed, above-label octreotide-LAR appears to be prescribed in patients with disease progression or uncontrolled symptoms while on standard dose therapy. Expert clinical opinion, as reported in this review, supports escalation of SSAs for patients with refractory hormonal symptoms. However, given limited published information on this topic, the safety and efficacy of above-label doses and/or frequency of octreotide-LAR in treatment of NETs warrants further evaluation.

COMMENTS

Background

Gastrointestinal neuroendocrine tumors (NETs) are rare and generally slow-growing neoplasms that secrete various peptides and neuroamines, some of which cause clinical symptoms. These tumors may be classified in multiple ways but are often divided into carcinoid tumors and pancreatic neuroendocrine tumors. First-line systemic therapy for NETs often consists of somatostatin analogs (SSAs) such as octreotide acetate or lanreotide.

Research frontiers

The maximum Food And Drug Administration-approved dosage and administration of octreotide long-acting repeatable (LAR), indicated for severe diarrhea/flushing episodes associated with metastatic carcinoid tumors and VIPomas, is 30 mg every 4 wk. However, in clinical practice, octreotide-LAR is sometimes prescribed at above-label doses but evidence for this practice has not been systematically assessed.

Innovations and breakthroughs

This systematic literature review suggests that above-label doses of octreotide-LAR are being used frequently for the management of NETs in clinical practice and excess toxicity was not observed in the reviewed studies. In most articles reviewed, above-label octreotide-LAR appears to be prescribed in patients with disease progression or uncontrolled symptoms while on standard dose therapy. Expert clinical opinion, as reported in this review, supports escalation of SSAs for patients with refractory hormonal symptoms. However, given limited published information on this topic, the safety and efficacy of above-label doses and/or frequency of octreotide-LAR in treatment of NETs warrants further evaluation.

Applications

The strength of this study lies in its comprehensive search, review, and synthesis of global literature on above the FDA-approved label dosage and frequency of administration of octreotide-LAR in patients with NETs. Thus, the findings summarized in this review may inform clinicians who manage patients with NETs and motivate future research in this field.

Peer-review

This is an informative, well conducted and documented review about a focused topic on the use of above-label doses of octreotide-LAR in patients with NETs.

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SYSTEMATIC REVIEWS

Distinctive inflammatory bowel disease phenotype in primary sclerosing cholangitis

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Author contributions: de Vries AB and Janse M performed the research, analyzed the data, and wrote the manuscript; Blokzijl H and Weersma RK were responsible for the study concept and design and were involved in editing and revision of the manuscript; all authors gave their final approval of the published version.

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Abstract

AIM: To review the current literature for the specific clinical characteristics of inflammatory bowel disease (IBD) associated with primary sclerosing cholangitis (PSC).

METHODS: A systematical review for clinical characteristics of IBD in PSC was performed by conducting a broad search for "primary sclerosing cholangitis" in Pubmed. "Clinical characteristics" were specified into five predefined subthemes: epidemiology of IBD in PSC, characteristics of IBD in PSC (*i.e.*, location, disease behavior), risk of colorectal cancer development, IBD recurrence and *de novo* disease after liver transplantation for PSC, and safety and complications after proctocolectomy with ileal pouch-anal anastomosis. Papers were selected for inclusion based on their relevance to the subthemes, and were reviewed by two independent reviewers. Only full papers relevant to PSC-IBD were included. Additionally the references of recent reviews for PSC (< 5 years old) were scrutinized for relevant articles.

RESULTS: Initial literature search for PSC yielded 4704 results. After careful review 65 papers, comprising a total of 11406 PSC-IBD patients, were selected and divided according to subtheme. Four manuscripts overlapped and were included in two subthemes. Prevalence of IBD in PSC shows a large variance, ranging from 46.5% to 98.7% with ulcerative colitis (UC) being the most common type (> 75%). The highest IBD rates in PSC are found in papers reviewing both endoscopic and histological data for IBD diagnosis. Although IBD in PSC is found to be a quiescent disease, pancolitis occurs often, with rates varying from 35% to 95%. Both backwash ileitis and rectal sparing are observed infrequently. The development of dysplasia or colorectal carcinoma is increased in PSC-IBD; the cumulative 10 years risk varying between 0% and 11%. Exacerbation of IBD is common after liver transplantation for PSC and *de novo* disease is seen in 1.3% to 31.3% of PSC-IBD patients. The risk for development of pouchitis in PSC-IBD is found to be

significant, affecting 13.8% to 90% of the patients after proctocolectomy with ileo anal-pouch anastomosis.

CONCLUSION: IBD in primary sclerosing cholangitis represents a distinct phenotype that differs from UC and Crohn's disease and therefore requires specialized management.

Key words: Primary sclerosing cholangitis; Inflammatory bowel disease; Incidence; Clinical characteristics; Risk of colorectal carcinoma; Liver transplantation; Pouchitis

Core tip: Inflammatory bowel disease (IBD) in primary sclerosing cholangitis (PSC) is observed to have different characteristics in comparison with conventional IBD without PSC. Based on these differences a distinct phenotype of IBD is suspected. Existing literature was reviewed for clinical characteristics of IBD in PSC yielding 65 studies consisting of 11406 PSC-IBD patients. A large variation for reported characteristics was found, which seem to be related to case ascertainment. This emphasizes the importance of full colonoscopy and biopsies to accurately diagnose IBD in PSC. Overall however, IBD in PSC shows large differences in comparison with conventional IBD, therefore representing a distinct phenotype.

de Vries AB, Janse M, Blokzijl H, Weersma RK. Distinctive inflammatory bowel disease phenotype in primary sclerosing cholangitis. *World J Gastroenterol* 2015; 21(6): 1956-1971 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i6/1956.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i6.1956

INTRODUCTION

Primary sclerosing cholangitis (PSC) is a chronic cholestatic liver disease characterized by inflammation and fibrosis of both the intrahepatic and extrahepatic bile ducts[1]. There is no curative therapy available and in most cases, PSC leads to liver cirrhosis and liver failure ultimately requiring liver transplantation[2,3]. In the United States and Western Europe, the reported incidence of PSC is 0.9 and 1.3 per 100.000/year, and the prevalence is 8.5 and 13.6 per 100000[4,5]. There is a strong relationship between PSC and inflammatory bowel disease (IBD). Approximately 50% to 80% of the patients with PSC are also diagnosed with IBD, finding ulcerative colitis (UC) in approximately 80% of these cases and Crohn's disease (CD) or unclassified IBD (IBD-U) in the remaining 20%[6]. In patients with IBD, PSC is found much less common. Approximately 2.4% to 7.5% of UC patients and 3.4% of CD patients are diagnosed with PSC[7]. With the established relationship between PSC and IBD[1,8], several studies aimed to characterize the clinical course and features of concomitant IBD and its differences from conventional UC and CD[9-12]. These studies showed increasing evidence that PSC-IBD may represent a distinctive phenotype in addition to the established and defined phenotypes UC and CD[11]. The IBD in PSC is frequently reported as a pancolitis, relatively often with rectal sparing. Although colitis in PSC tends to follow a quiescent course, patients with PSC-IBD are found to have a relatively high risk of developing colorectal carcinoma compared to IBD patients without PSC[13]. However, while evidence for distinct PSC-IBD phenotype is increasing, its reported characteristics vary. In this systematic review of the literature we therefore aimed to describe the specific characteristics of the PSC-IBD phenotype based on the most recent literature. We reviewed prevalence of IBD in PSC, its phenotypic characteristics and the risk of development of colorectal malignancy. In addition we looked at IBD associated with PSC in relation to its clinical course or *de novo* presentation after orthotopic liver transplantation (OLT) and we reviewed the safety of proctocolectomy and incidence of pouchitis reported for PSC-IDB. Finally, we discussed the shared and non-shared genetic factors to further highlight the distinct nature of this phenotype. Insight into how PSC-IBD differs from general IBD, will help clinicians in the field of IBD in their approach for this specific group of patients.

MATERIALS AND METHODS

We first defined relevant subthemes related to the subject of IBD in PSC based on clinical observations and recent literature.

Themes of interest

Epidemiology of IBD in PSC: Reported incidence, methods of case ascertainment and geographical variation.

Phenotypic features of IBD in PSC: Disease localization, activity, and its association with backwash ileitis and rectal sparing.

Colorectal cancer risk in PSC-IBD: The incidence and risk in comparison with conventional IBD.

PSC-IBD in relation to liver transplantation: Disease behavior after OLT and the occurrence of *de novo* IBD.

Ileal pouch anal anastomosis in PSC-IBD: Safety of the procedure and pouch complications.

A broad literature search using the keyword "primary sclerosing cholangitis" was performed in PubMed (http://www.ncbi.nlm.nih.gov/pubmed),

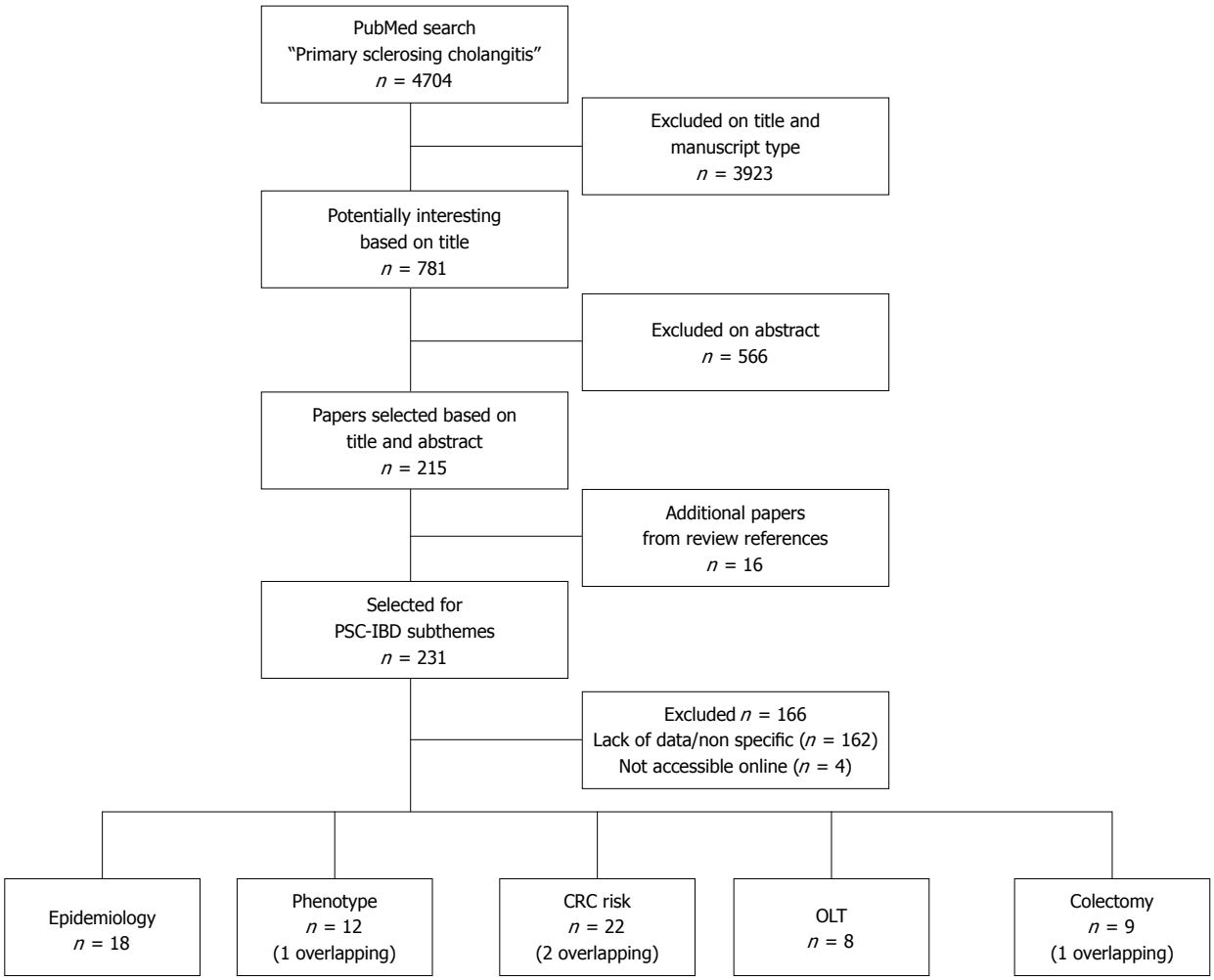

Figure 1 Flow chart paper inclusion: An overview of study selection for papers included in the present review. PSC: Primary sclerosing cholangitis; IBD: Inflammatory bowel disease; CRC: Colorectal cancer; OLT: Orthotopic liver transplantation.

an online database comprising more than 23 million citations for biomedical literature from MEDLINE, life sciences journals, and online books. Relevant papers addressing the defined subthemes were selected based on title and abstract. Papers not found relevant by both reviewers (BdV and MJ) were excluded. When in disagreement, papers were discussed and included or excluded based on mutual agreement. We only included full papers relevant to PSC-IBD. Additionally the references of recent review papers (< 5 years) concerning PSC-IBD were scrutinized for relevant articles. Full papers of the selected abstracts were reviewed by BdV. References in manuscripts specifically assessing the IBD phenotype in PSC were hand-searched to identify other relevant papers.

RESULTS

The literature search on "PSC" yielded 4704 results. After reviewing titles and manuscript type (excluding reviews), a total of 781 papers were selected for assessment of their abstracts. After careful review, 215 papers were selected and divided into a specific subtheme. The scrutinizing of references from reviews and PSC-IBD manuscripts yielded another 16 papers. After reading the full text, 65 papers, comprising a total of 11406 patients with PSC-IBD, were selected for the review (Figure 1). Criteria for study selection were study size, relevance to the subthemes, and specification of data (*i.e.,* number of patients with backwash ileitis, localization of IBD). Four papers[11,14-16] overlapped, and were included in two subthemes.

Epidemiology: Reported incidence and prevalence of IBD in PSC

Literature search and evaluation of reviews yielded 18 papers with a total of 6589 included PSC-IBD patients. Data concerning epidemiology of PSC and associated IBD are summarized in Table 1.

PSC is a rare disease with a reported incidence between 0.07 and 1.31 per 100000[4,17]. The prevalence of associated IBD ranged from 46.5%[17] to 98.7%[18], with UC being the most common type (> 75%) in PSC-IBD (Table 1). CD in PSC-IBD is found in approximately 16% of PSC patients (range:

Table 1 Epidemiology primary sclerosing cholangitis - inflammatory bowel disease *n* (%)

Ref.	Country	Period	Incidence[1]	PSC	Male Gender	PSC-IBD	PSC-UC	PSC-CD	PSC-IBD-U	Diagnosis IBD
Rabinovitz *et al*[100], 1990	United States	NA	NA	66	NA	47 (71.2)	39 (83.0)	8 (17.0)	0	IBD[2,3]
Farrant *et al*[19], 1991	United Kingdom	1972-1989	NA	126	78 (61.9)	85 (67.5)	83 (97.6)	2 (2.4)	NA	IBD[2]
Schrumpf *et al*[18], 1994	Norway	1975-1989	NA	77	51 (66.2)	76 (98.7)	58 (76.3)	11 (14.5)	7 (9.2)	IBD[2,3]
Escorsell *et al*[17], 1994	Spain	1984-1988	0.07	43	26 (60.5)	20 (46.5)	19 (95.0)	1 (5.0)	NA	IBD[4]
Broomé *et al*[2], 1996	Sweden	? - 1992	NA	305	195 (63.9)	249 (81.6)	220 (88.4)	20 (8.0)	9 (3.6)	IBD[2]
Boberg *et al*[4], 1998	Norway	1986-1995	1.31	17	12 (70.6)	12 (70.6)	9 (75.0)	2 (16.7)	1 (8.3)	IBD[4]
Ponsioen *et al*[20], 2002	The Netherlands	1979-1999	NA	174	105 (60.3)	114 (65.5)	83 (72.8)	28 (24.6)	3 (2.6)	IBD[4]
Bambha *et al*[5], 2003	United States	1976-2000	0.90	22	15 (68.3)	16 (72.7)	12 (75.0)	3 (18.8)	1 (5.0)	IBD[2]
Kingham *et al*[101], 2004	United Kingdom	1984-2003	0.91	53	33 (62.3)	33 (62.3)	30 (90.9)	3 (19.1)	0	IBD[4]
Bergquist *et al*[102], 2005	Sweden	1984-1999	NA	145	100 (69.0)	126 (86.9)	107 (84.9)	19 (15.1)	0	IBD[2,3]
Tischendorf *et al*[103], 2007	Germany	1978-2004	NA	273	195 (71.4)	172 (63.0)	141 (82.0)	29 (16.9)	2 (1.2)	IBD[2,3]
Bergquist *et al*[104], 2007	Sweden	1984-2004	NA	246	165 (67.1)	195 (79.3)	166 (85.1)	21 (10.8)	8 (4.1)	IBD[2,3]
Kaplan *et al*[14], 2007	Canada	2000-2005	0.92	49	27 (55.1)	36 (73.5)	17 (47.2)	19 (52.8)	0	IBD[2,3]
Card *et al*[3], 2008	United Kingdom	1987-2002	0.41	223	141 (63.2)	108 (48.4)	67 (62.0)	13 (12.0)	28 (25.9)	IBD[4]
Lindkvist *et al*[105], 2010	Sweden	1992-2005	1.22	199	142 (71.4)	152 (76.4)	129 (84.9)	17 (11.2)	5 (3.3)	IBD[4]
Bowlus *et al*[106], 2010	United States	1995-2009	NA	6767	4475 (66.1)	4637 (68.5)	3067 (66.1)	1090 (23.5)	480 (10.4)	IBD[4]
Toy *et al*[107], 2011	United States	2000-2006	0.41	169	101 (59.8)	109 (64.5)	95 (87.2)	13 (11.9)	1 (0.9)	IBD[4]
Boonstra *et al*[22], 2013	The Netherlands	2000-2007	0.50	590	375 (63.6)	402 (68.1)	308 (76.6)	78 (19.4)	16 (4.0)	IBD[2,3]

[1]Incidence for PSC per 100000 people; [2]IBD Endoscopic findings; [3]IBD Histological findings (biopsies); [4]IBD diagnosis not specified/medical records only. PSC: Primary sclerosing cholangitis; UC: Ulcerative colitis; CD: Crohns disease; IBD-U: Inflammatory bowel disease unclassified; NA: Not available.

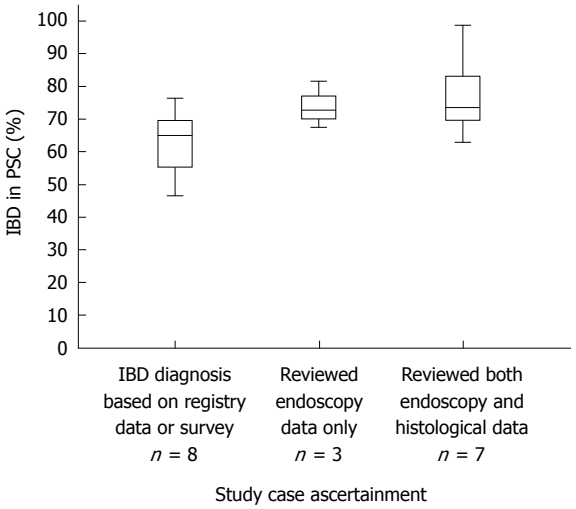

Figure 2 Case ascertainment: A comparison of included primary sclerosing cholangitis - inflammatory bowel disease epidemiology studies based on case ascertainment. PSC: Primary sclerosing cholangitis; IBD: Inflammatory bowel disease.

2.4%[19]-24.8%[20]). IBD-U is the least common IBD-type in PSC, seldom representing more than 5%. The actual percentages are expected to be even lower, as studies on IBD-U with and without associated PSC show that part of the initial IBD-U diagnosis is later re-classified as either UC or CD[15,21].

Varying IBD rates found in PSC: A recent large Dutch population-based study showed lower incidence rates for both PSC and PSC-IBD when compared with most other studies[22]. This emphasizes the importance of large multi-center studies with proper case ascertainment in studying rare diseases. In this review, all but two of the selected studies concerning epidemiology reviewed cholangiography or MRCP imaging to confirm the PSC diagnosis. Endoscopic imaging was reviewed in ten (55.5%) studies, of which seven also reviewed histological data to ascertain the method of IBD diagnosis in the included patients (Table 1). In the remaining eight studies, the presence of IBD in PSC was established or excluded based on registry data, physician surveys, or notes in medical file without reviewing original endoscopy or histology. Combined study population of these groups consisted of 1446 (endoscopy and histology), 453 (endoscopy only) and 7645 (no review of original endoscopy or histology) PSC patients respectively. The studies that used both endoscopic and histological confirmation for IBD diagnosis seem to show a higher median percentage of IBD in PSC when compared with studies that only used endoscopy or did not review diagnostic workup for IBD (Figure 2).

Geographic variation IBD - PSC: Geographic variation in the incidence of PSC and associated IBD has been reported, with highest rates found in Western Europe and North America (Table 1) and lower rates in Asia[23-25]. Several studies from Asia reported an incidence of IBD in PSC ranging between 20% and 34%[24,26-28].

Key points: Approximately 70% of patients with PSC have associated IBD, predominantly UC. The highest rates of IBD in PSC were observed in papers using strict case ascertainment criteria. Large geographic differences in the incidence of PSC and associated

Table 2 Phenotypic features primary sclerosing cholangitis - inflammatory bowel disease *n* (%)

Ref.	IBD (*n*)	PSC-IBD (*n*)	Proctitis		Leftsided		Pancolitis		Backwash		Rectal Sparing		Diagnosis IBD
			IBD	PSC-IBD	IBD	PSC-IBD	IBD	PSC-IBD	IBD	PSC-IBD	IBD	PSC-IBD	
Olsson *et al*[9], 1991	1445	55	552 (38.2)	3 (5.5)	NA	NA	893 (61.8)	52 (94.5)	NA	NA	NA	NA	IBD[1,2]
Loftus *et al*[11], 2005	142	71	NA	NA	NA	NA	76 (53.5)	60 (84.5)	10 (7.0)	20 (28.2)	8 (5.6)	34 (47.9)	IBD[1,2]
Kaplan *et al*[14], 2007	0	36	NA	NA	NA	NA	NA	17 (47.2)	NA	4 (11.1)	NA	2 (5.6)	IBD[1,2]
Sokol *et al*[15], 2008	150	75	138 (92.0)	68 (90.7)	130 (86.7)	68 (90.7)	91 (60.7)	49 (65.3)	36 (24.0)	14 (18.7)	20 (13.3)	15 (20.0)	IBD[1,2]
Joo *et al*[31], 2009	40	40	0	0	14 (35.0)	3 (7.5)	18 (45.0)	34 (85.0)	3 (7.5)	4 (10.0)	10 (25.0)	11 (27.5)	IBD[1,2]
Sano *et al*[32], 2010	60	20	18 (30.0)	1 (5.0)	19 (31.7)	1 (5.0)	21 (35.0)	7 (35.0)	NA	NA	NA	NA	IBD[1,2]
Ye *et al*[25], 2011	63	21	NA	NA	NA	NA	35 (55.6)	20 (95.2)	2 (3.2)	9 (42.9)	1 (1.6)	8 (38.1)	IBD[1,2]
Jørgensen *et al*[33], 2012	0	110	NA	NA	NA	3 (2.7)	NA	60 (54.5)	NA	17 (15.5)	NA	73 (66.4)	IBD[1,2]
O'toole *et al*[35], 2012	2649	103	209 (7.9)	1 (1.0)	649 (24.5)	23 (22.3)	663 (25.0)	56 (54.4)	NA	NA	NA	NA	IBD[1,2]
Boonstra *et al*[12], 2012	0	380	NA	9 (2.4)	NA	34 (8.9)	NA	219 (57.6)	NA	NA	NA	NA	IBD[1,2]
Boonstra *et al*[12], 2012[3]	80	80	4 (5.0)	2 (2.5)	16 (20.0)	2 (2.5)	35 (43.8)	52 (65.0)	2 (2.5)	4 (5.0)	1 (1.3)	8 (10.0)	IBD[1,2]
Schaeffer *et al*[34], 2013	0	97	NA	0	NA	17 (17.5)	NA	42 (43.3)	NA	NA	NA	NA	IBD[1,2]
Sinakos *et al*[30], 2013	0	129	NA	NA	NA	16 (12.4)	NA	76 (58.9)	NA	15 (11.6)	NA	31 (24.0)	IBD[1,2]
Mean			28.8%	13.4%	39.6%	18.8%	47.5%	64.7%	12.3%	16.7%	9.9%	30.9%	

[1]IBD Endoscopic findings; [2]IBD Histological findings (biopsies); [3]Subgroup analysis. PSC: Primary sclerosing cholangitis; IBD: Inflammatory bowel disease; NA: Not available.

IBD between east and west are reported.

Distinctive features of IBD in PSC

Frequently documented characteristics of IBD in PSC include a mild or quiescent disease course and a high rate of pancolitis[9,29,30]. The association with backwash ileitis and rectal sparing in PSC-IBD is also suggested, but less consistently. The present review included twelve studies (1217 patients with PSC-IBD) describing features of IBD in PSC, which are summarized in Table 2. In seven (58.3%) studies a comparison with conventional IBD was made.

Disease activity: Out of the twelve included studies, eight (66.6%) characterized the clinical course of IBD in PSC. All eight studies classified the IBD's overall activity as mild[9,11,15,30-34]. Every included study reviewed endoscopic and histological data to confirm IBD diagnosis (Table 2). In addition, four studies also assessed histological severity of inflammation in different segments of the colon, none finding severe inflammation in PSC-IBD[31-34]. The majority of these studies found that prevalence for inflammation was highest in the right-sided colon and lowest toward the distal colon. Both Joo *et al*[31] and Sano *et al*[32] reported this was significantly different (0.019 and 0.034 respectively) in matched IBD-controls, with histologically graded inflammation being highest in the proximal colon and lowest in the rectum for PSC-IBD. In accordance with these results, two studies[12,33] reported a significant predominance of right-sided inflammation in PSC-IBD observed during endoscopy.

Disease localization in PSC-IBD: Localization and extent of IBD were determined by endoscopy in the majority of studies (Table 2). Involvement of the whole colon is more common in PSC-IBD (35%[32]-95%[25], mean 64.7%) than in IBD controls (25%[35]-62%[9], mean 47.5%). Several authors have found these differences to be significant[11,12,25]. The reported rates of left sided colitis in PSC-IBD varies between studies (2.5%[12]-90.7%[15], mean 18.8%), but are lower than in IBD without concomitant PSC (20%[12]-86.7%[15], mean 39.6%). The overall reported occurrence of proctitis-only is low for PSC-IBD in most studies (1%[35]-5.5%[9], Table 2) and less common in comparison with IBD controls (5%[12]-38%[9]).

Backwash ileitis: Backwash ileitis (BWI) is an inflammation of the ileum due to diminished ileocecal valve function in severe UC, allowing for retrograde flow of colonic content and inflammation of the ileum[36]. The reported rates of BWI found for PSC-UC range between 5.0%-42.9% (mean 16.7%). In UC without PSC involvement of the ileum is infrequent, with reported rates ranging from 2.5%[12] to 24%[15] (mean 12.3%, Table 2).

Rectal sparing: Rectal sparing (RS) is described as rectal mucosa in UC or CD which is completely or partially spared from active or chronic inflammation in comparison to the more proximal colon[37]. The frequency of RS in PSC-IBD is specified in eight studies (Table 2). The reported incidence shows a large variation ranging from 5.6%[14] and 66.4%[33] (mean 30.9%) in PSC-IBD, compared with 1.6%[25] and 25%[31] (mean 9.9%) in IBD without PSC.

PSC-CD: The localization of PSC-CD is specified in five studies[11,12,14], two of which focused on the PSC-CD phenotype[38,39]. Colonic involvement in PSC-CD is reported most often (36.8%[14]-82.1%[38] followed by involvement of both ileum and colon (21.8%[12]-57.9%[14]). Isolated ileal involvement is

rare in PSC-CD (2%[39]-5%[12]). The rate of ileitis is similar or lower in PSC-CD compared with CD controls[12,38,39]. Rectal sparing is not significantly increased in PSC-CD[12,38] and isolated upper gastrointestinal CD is reported in only two patients[15,39]. The disease activity in PSC-CD is characterized as similar or less active then in CD controls, with lower rates of stricturing and penetrating disease seen during endoscopy[12,38,39].

Key points: IBD associated with PSC is characterized by a quiescent course with a predominance for mild right-sided inflammation and a proximal- or pancolitis. Although less common than pancolitis, both backwash ileitis and rectal sparing are more often found in PSC-UC compared with UC. In addition, strictures and penetrating disease were found to be less common in PSC-CD than in CD without PSC.

Colorectal cancer risk in PSC-IBD

The increased risk of development of colorectal cancer (CRC) in IBD has been widely recognized for several decades[40-44]. Both disease extent and duration of IBD have been identified as factors associated with this increased cancer risk[41,43,44]. An additional risk for development of dysplasia and CRC in PSC-IBD was observed by several authors[45-48]. A total of twenty-two studies assessing CRC risk in PSC-IBD were reviewed, comprising a total of 2936 patients with PSC-IBD (Table 3).

CRC incidence and location in PSC-IBD: The 10-year cumulative risk for the development of colonic dysplasia and CRC in PSC-IBD is reported in five studies[45,49-52] and varies between 0%[49] and 11%[50]. In these studies the data on dysplasia or CRC development for IBD without PSC is limited, showing a 10 year cumulative risk of approximately 2%[45,50,51]. The observed percentage of included PSC-IBD patients developing dysplasia and CRC shows large differences, ranging from 1.3%[53] to 66.7%[54]. For the IBD controls, these percentages vary from 0.7%[55] to 30.1%[46]. The mean time interval between diagnosis of IBD and development of CRC in PSC-IBD ranges between 12.2[49] and 20[56] years (Table 3). In comparison, in IBD without PSC the mean interval varies between 17.5[50] and 44.4[15] years. The median age at which dysplasia or CRC presented itself was specified in three studies on PSC-IBD and in one study on IBD, being lower in the former (Table 3). For IBD the development of CRC in the recto-sigmoid has been described more common in UC whereas CD shows a more equal distribution between right sided and left sided colonic malignancy[57]. Dysplasia or CRC in PSC-IBD frequently develops in the proximal colon with reported rates varying between 28.6%[11] and 100%[55]. For IBD a proximal development of CRC and dysplasia is found less common (0%[15]-50%[11], Table 3).

CRC in PSC-CD and PSC without IBD: Limited data is available on the risk of CRC development in PSC-CD patients as it is less prevalent, and often only represents a few cases within PSC-IBD cohorts. Furthermore, disease characteristics of IBD in PSC can make it difficult to distinguish between UC and CD, resulting in a change of diagnosis during follow-up. A study by Lindström *et al*[38], which compared 28 PSC-CD patients with 46 CD controls found an increased risk of CRC development in PSC-CD that was comparable to the risk in PSC-UC. In contrast, a study by Braden *et al*[58] found a lower risk in PSC-CD compared with PSC-UC by looking at CRC development in 166 PSC-IBD (35 PSC-CD) and 216 IBD controls (102 CD). Risk of CRC development in PSC patients without IBD was found to be low[22,29,48,52].

Key points: The development of dysplasia and CRC in PSC-IBD is increased and has predominance for right-sided localization in comparison to conventional IBD. Based on limited results, presentation of dysplasia or CRC in PSC-IBD at a younger age is suggested. It remains unclear whether the interval between IBD diagnosis and dysplasia or CRC development was shorter.

PSC-IBD in relation to liver transplantation

In PSC patients after orthotopic liver transplantation (OLT), IBD exacerbations and even development of *de novo* IBD have been reported by a number of studies, despite continuous use of immunosuppressive medication[59-62]. To review IBD disease activity and *de novo* presentation in PSC-IBD after OLT, eight studies comprising 519 patients with PSC-IBD were included. Papers concerning both autoimmune hepatitis and PSC were scrutinized for PSC-IBD data only. An overview of the selected papers is presented in Table 4.

IBD Exacerbations and *de novo* disease after OLT for PSC: Exacerbation of IBD after OLT is estimated to occur in 8.6%[63] to 51.5%[62] of the patients, whereas *de novo* IBD is seen less frequently, varying from 1.3%[64] to 31.3%[61] in patients transplanted for PSC. Long term outcome for IBD in PSC is reported in seven studies, with colectomy rates after OLT varying from 4.3%[59] to 19.5%[65] (Table 4). Treatment-refractory IBD as an indication for colectomy ranged from 0%[59] to 85.7%[60].

Key points: Approximately 32.6% of patients with PSC-IBD experience an exacerbation of IBD after their liver transplantation. In addition, approximately 18.3% of patients develop *de novo* IBD after liver

Table 3 Dysplasia or colorectal cancer in primary sclerosing cholangitis - inflammatory bowel disease *n* (%)

Ref.	IBD (*n*)	PSC-IBD (*n*)	Country	Dysplasia		Risk/incidence		Proximal colon[1]		Age dysplasia diagnosis (yr)		IBD duration (yr)		Diagnosis IBD
				IBD	PSC-IBD	IBD	PSC-IBD	IBD	PSC-IBD	IBD	PSC-IBD	IBD	PSC-IBD	
Broomé *et al*[45], 1995	80	40	Sweden	7 (8.8)	11 (27.5)	CR = 10 yr: 2%	CR = 10 yr: 9%	NA	NA	NA	NA	26.0[2]	18.0[2]	IBD[3,4]
Gurbuz *et al*[49], 1995	0	35	United States	NA	13 (37.1)	NA	CR = 10 yr: 0%	NA	NA	NA	51.4[2]	NA	12.2[2]	IBD[3,4]
Brentnall *et al*[56], 1996	25	20	United States	4 (16)	9 (45)	NA	OR = 4.9	NA	NA	NA	NA	NA	20.0[2]	IBD[3,4]
Loftus *et al*[29], 1996	0	143	United States	NA	27 (18.9)	NA	CR = 20 yr: 17%	NA	42.9%[5]	NA	41.0[6]	NA	NA	IBD[3,4]
Kornfeld *et al*[48], 1997	0	58	Sweden	NA	5 (8.6)	NA	NA	NA	NA	NA	NA	NA	NA	IBD[7]
Leidenius *et al*[50], 1997	45	45	Finland	4 (8.9)	13 (28.9)	CR = 10 yr: 3%	CR = 10 yr: 11%	NA	NA	NA	NA	17.5[2]	11.0[6]	IBD[3,4]
Marchesa *et al*[54], 1997[8]	1185	27	United States	145 (12.2)	18 (66.7)	NA	RR = 10.4	40.8%[5]	100.0%[5]	NA	NA	NA	NA	IBD[4]
Shetty *et al*[84], 1999	196	132	United States	11 (5.6)	33 (25)	RR = 1	RR = 3.15	20.0%[5]	76.5%[5]	NA	NA	NA	NA	IBD[3,4]
Lindberg *et al*[46], 2001	103	19	Sweden	31 (30.1)	12 (63.2)	CR = 20 yr: 16%	CR = 20 yr: 32%	32.3%	69.2%	NA	NA	NA	NA	IBD[3,4]
Bergquist *et al*[108], 2002	0	477	Sweden	NA	35 (7.3)	NA	SIR = 10.3	NA	NA	NA	NA	NA	NA	IBD[3,4]
Loftus *et al*[11], 2005	142	71	United States	15 (10.6)	18 (25.4)	CP = 10 yr: 20%	CP = 10 yr: 53%	50%[5]	28.6%[5]	NA	NA	12.1[6]	12.7[6]	IBD[3,4]
Kaplan *et al*[109], 2007	0	45	Canada	NA	5 (11.1)	NA	IR = 3.1	NA	NA	NA	NA	NA	NA	IBD[3,4]
Lepistö *et al*[16], 2008	389	52	Finland	63 (16.2)	17 (32.7)	CR = 20 yr: 0.39	CR = 20 yr: 0.43	NA	NA	NA	NA	NA	NA	IBD[4]
Sokol *et al*[15], 2008	150	75	France	2 (1.3)	10 (13.3)	CR = 25 yr: 1.5%	CR = 25 yr: 25.6%	0.0%[5]	100.0%[5]	NA	NA	44.4[2]	17.4[2]	IBD[3,4]
Terg *et al*[51], 2008	64	39	Argentina	2 (3.1)	7 (17.9)	CR = 10 yr: 2.0%	CR = 10 yr: 11%	NA	NA	NA	NA	NA	NA	IBD[3,4]
Claessen *et al*[52], 2009	0	126	The Netherlands	NA	35 (27.8)	NA	CR = 10 yr: 9%	NA	63.0%[5]	NA	NA	NA	12.6[6]	IBD[3,4]
Lindström *et al*[38], 2011	46	28	Sweden	3 (6.5)	9 (32.1)	NA	OR = 6.78	0.0%	66.0%	NA	NA	10.0[6]	12.0[6]	IBD[3,4]
Braden *et al*[58], 2012	216	166	United Kingdom	14 (6.5)	14 (8.4)	CIR = 2.9%	CIR = 7.5%	30.0%	35.7%	NA	NA	NA	NA	IBD[3,4]
Imam *et al*[53], 2012	0	784	United States	NA	10 (1.3)	NA	I = 0.4/yr	NA	60.0%	NA	37.4[2]	NA	NA	IBD[3,4]
Jess *et al*[55], 2012	47374	NA	Denmark	329 (0.7)	9 (?)[9]	RR = 1.07 (UC)	RR = 9.13	38.0%	100.0%	71.66	64.0[6]	NA	13.7[6]	IBD[7]
de Valle *et al*[79], 2012	0	152	Sweden	NA	3 (2.0)	NA	SIR = 4.31	NA	NA	NA	NA	NA	NA	IBD[3,4]
Boonstra *et al*[22], 2013	722	402	The Netherlands	7 (1)	19 (4.7)	SIR = 1.2	SIR = 8.6	NA	NA	596	39.0[6]	4.0[6]	15.0[6]	IBD[3,4]

[1]From cecum to splenic flexura; [2]Mean; [3]Endoscopic findings; [4]Histological findings (biopsies); [5]For CRC only; [6]Median; [7]Unknown or not reviewed; [8]Abstract; [9]Population based study, amount of PSC-IBD patients not specified. RR: Relative risk; CR: Cumulative risk; CP: Cumulative probability; CIR: Cumulative incidence rate; SIR: Standard incidence ratio; OR: Odds ratio; IR: Incidence rate; I: Incidence; NA: Not available; PSC: Primary sclerosing cholangitis; IBD: Inflammatory bowel disease.

transplantation for PSC.

PSC-IBD in relation to proctocolectomy

Restorative proctocolectomy with ileal pouch anal anastomosis (IPAA) is performed for colonic dysplasia or treatment-refractory disease in UC with and without PSC. A long term complication after IPAA is the development of an acute or chronic inflammation of the ileal pouch[66]. Several reports have associated PSC-UC with a more frequent development of pouchitis in comparison with UC without liver involvement[67-70]. We reviewed nine studies that researched pouchitis in PSC-IBD after proctocolectomy, comprising a total of 379 PSC-IBD patients with IPAA (Table 5).

Pouchitis incidence: The reported overall incidence of pouchitis (ranging from an isolated episode to chronic pouchitis) for PSC-UC ranges from 13.8%[71] to 90%[68] compared with 11.9%[71] to 52.8%[72] in UC without PSC. In addition, the number of patients with chronic pouchitis in PSC-IBD varies from 13.8%[71] to 70%[68]. Chronic pouchitis is found in 9.6%[72] to 15%[69] of the IBD patients after proctocolectomy and IPAA. Apart from the incidence of pouchitis, pouch failure

Table 4 Primary sclerosing cholangitis - inflammatory bowel disease and liver transplantation *n* (%)

Ref.	*n*	Pre-OLT			Post-OLT				
		PSC only	PSC-IBD	Intact colon[1]	*De novo* IBD	Exacerbation	Colectomy	Refractory IBD	Median follow-up (yr)
Dvorchik *et al*[65], 2002	192	0	192	169 (88.0)	NA	22 (13.0)	33 (19.5)	22 (66.7)	5.9[3]
Haagsma *et al*[59], 2003	48	25 (52.1)	23 (47.9)	23 (100.0)	6 (24.0)	9 (39.1)	1 (4.3)	0	7.2
Verdonk *et al*[88], 2006	60	15 (25.0)	45 (75.0)	45 (100.0)	3 (20.0)	NA	NA	NA	6.1
Cholongitas *et al*[62], 2007[2]	56	18 (32.1)	38 (67.9)	33 (68.8)	3 (16.6)	17 (51.5)	7 (16.7)	3 (42.9)	2.8
Moncrief *et al*[61], 2010	59	16 (27.1)	42 (71.2)	32 (76.2)	5 (31.3)	13 (40.6)	6 (18.8)	4 (66.7)	5.6
Joshi *et al*[60], 2011	110	36 (32.7)	74 (67.3)	65 (87.8)	6 (16.7)	33 (50.8)	7 (10.8)	6 (85.7)	6.5[3]
Navaneethan *et al*[63], 2012	77	0	77	58 (75.3)	NA	5 (8.6)	9 (15.5)	3 (33.3)	5.0
Mosli *et al*[64], 2013	105	77 (73.3)	28 (26.7)	24 (85.7)	1 (1.3)	6 (25.0)	2 (8.3)	0	7.3[3]

[1]Patients with PSC-IBD and without proctocolectomy prior to OLT; [2]Study population corrected for survival > 1 year after OLT (*n* = 56, 18 PSC only, 33 PSC-IBD, 5 PSC-IBD with pre-OLT colectomy, extracted from paper); [3]Mean instead of median. OLT: Orthotopic liver transplantation; PSC: Primary sclerosing cholangitis; IBD: Inflammatory bowel disease; NA: Not available.

during follow up was assessed in five studies, which all showed similar results (Table 5). Pouch failure was seen in 1.5%[71] to 16.1%[70] of the PSC-IBD patients and in 3.3%[16] to 11.2%[72] of the IBD patients after IPAA. Safety of proctocolectomy with IPAA in PSC-IBD was discussed in three studies, with the majority describing it as a safe procedure[73,74].

Key points: Pouchitis after IPAA is more frequently found in PSC-IBD patients compared with IBD patients. Rate of pouch failure seems similar for IBD patients with and without PSC. Although data are limited, proctocolectomy with IPAA is deemed safe in PSC-IBD patients by the majority of studies.

DISCUSSION

In this review we have characterized the clinical features of IBD associated with PSC. In addition, we aimed to provide data to assist clinicians in the IBD field, to aid management for this specific group. PSC-IBD can be said to represent a distinct phenotype that differs from both UC and CD.

The prevalence for IBD found in PSC is high, being present in approximately 70% of the cases, with a large variation found between studies. The most common type is UC which represents > 75% of IBD found in PSC, followed by the less common CD and infrequently seen IBD-U. In contrast, a relative high prevalence of CD was found in a study by Kaplan *et al*[14] reporting 38.8% CD in the total of included patients with PSC. As possible explanations the authors suggested an increasing trend of awareness of PSC in IBD other than UC and the high prevalence of CD found (1.5 times that of UC) in the province of Alberta, Canada. Geographic differences could explain the large variation found in IBD prevalence in PSC. We found a high prevalence of IBD in PSC patients (> 60%) in Europe and North America, compared with lower rates of approximately 30% IBD in PSC patients in Asia. In most studies from Asia on PSC-IBD however, endoscopic and histological data were not specified for the IBD diagnosis, making case ascertainment unreliable. A more recent Japanese study[32] establishing IBD diagnosis in PSC based on both endoscopic and histological data, showed an IBD incidence of 68%, similar to the percentage found in studies from Europe and North America. Although these results were based on a small study population and need to be validated by future large multicenter studies, they do suggest that case ascertainment affects the prevalence of IBD found in PSC. To explore whether reported rates of IBD in PSC are influenced by method of case ascertainment, a comparison between included epidemiological studies was made. Strikingly, the highest rates of IBD in PSC were found in studies using the most stringent criteria, reviewing both endoscopic and histology data, to exclude or establish the presence of IBD in PSC. In addition, the lowest prevalence of IBD in PSC was reported in studies using registry data, physician surveys or notes in the medical file without reviewing original endoscopy or histology. These results are in accordance with the observation that IBD in PSC can be present without clinical signs and with normal endoscopic appearance[33,75], resulting in underdiagnosis[45]. This observation stresses the importance of histological diagnosis in PSC-IBD. In contrast, one of the largest PSC population-based studies to date, shows a relatively low rate of IBD in PSC patients (68.1%), while reviewing both histology and endoscopy[22]. Our findings can therefore not fully explain the large variation found in IBD prevalence in PSC, but they do stress the importance of full endoscopy combined with random biopsies to determine the presence of IBD in PSC patients.

The present review found that IBD in PSC patients is characterized by a mild disease activity, seldom showing severe inflammation. The prevalence of colonic inflammation follows a proximal to distal distribution, with most activity found in the cecum and ascending colon. A pancolitis is seen in > 60% of IBD associated with PSC. Isolated distal colon involvement in PSC-IBD is seen in < 20%, with

Table 5 Pouchitis in primary sclerosing cholangitis - inflammatory bowel disease *n* (%)

Ref.	Country	IBD (*n*)	PSC-IBD (*n*)	IPAA		Pouchitis		Chronic pouchitis		Pouch failure		Diagnosis
				IBD	PSC-IBD	IBD	PSC-IBD	IBD	PSC-IBD	IBD	PSC-IBD	
Kartheuser *et al*[74], 1993	United States	NA	40	NA	40	NA	19 (47.5)	NA	NA	NA	NA	D[1]
Penna *et al*[69], 1996	United States	1043	54	1043	54	336 (32.2)	34 (63.0)	? (15.0)[2]	? (60.0)[2]	NA	NA	D[3]
Aitola *et al*[68], 1998	Finland	63	10	63	10	19 (30.2)	9 (90.0)	7 (11.1)	7 (70.0)	NA	NA	D[3]
Gorgun *et al*[71], 2005	United States	260	65	260	65	31 (11.9)	9 (13.8)	31 (11.9)	9 (13.8)	19 (7.3)	1 (1.5)	D[3,4,5]
Abdelrazeq *et al*[67], 2007	United Kingdom	182	16	182	16	53 (29.1)	11 (68.8)	18 (9.9)	9 (56.3)	NA	NA	D[3,4,5]
Lepistö *et al*[16], 2008	Finland	389	52	389	52	101 (26.0)	25 (48.1)	NA	NA	13 (3.3)	2 (3.8)	D[1]
Wasmuth *et al*[72], 2010	Norway	178	11	178	11	94 (52.8)	8 (72.0)	17 (9.6)	4 (36.4)	20 (11.2)	1 (9.1)	D[3,4]
Mathis *et al*[73], 2011	United States	NA	100	NA	100	NA	64 (64.0)	NA	16 (16.0)	NA	3 (3.0)	D[3,4,5]
Block *et al*[70], 2013	Norway	113	48	62	31	20 (32.3)	27 (87.1)	8 (12.9)	20 (64.5)	4 (6.5)	5 (16.1)	D[3,4,5]

[1]Not specified; [2]Not otherwise specified in paper; [3]Clinical diagnosis pouchitis; [4]Endoscopic diagnosis pouchitis; [5]Histological diagnosis pouchitis. IPAA: Ileal pouch anal anastomosis; PSC: Primary sclerosing cholangitis; IBD: Inflammatory bowel disease; NA: Not available; D: Diagnosis.

isolated proctitis being uncommon. IBD in PSC can therefore be characterized by pancolitis and or right-sided colon involvement. While the exact mechanism of this distribution is unknown, a possible contributing factor was proposed by Schaeffer *et al*[34], who found differences in localization based on initial disease presentation in PSC-IBD. PSC diagnosis followed by IBD development was more often associated with right sided colonic inflammation, while pancolitis was found more frequent in IBD developing before PSC diagnosis.

Although a relation between backwash ileitis (BWI) and PSC-UC has been documented, it is not undisputed. Some studies for example, reported high rates[11,25], which in turn could not be confirmed by other studies[12,31]. While more frequently found in PSC-UC compared with IBD without PSC, BWI seems to be an uncommon feature of PSC-UC as it is only found in approximately 16% of the patients. Comparison between rates observed in PSC-UC is complicated by a large variation found between studies, which can be explained by differences in BWI diagnosis based on definition. Some studies, for example, noted all histological changes of the ileum as BWI[11,25], while others specified between active inflammation and chronic inflammation and only used the former for the observed BWI rates[31]. Apart from differences in definition, the localization and degree of colonic inflammation have also been associated with prevalence of BWI. Haskell *et al*[36], reporting a 17% prevalence of BWI in UC (n = 200), noted the importance of correlating the presence of histological cecal colonic inflammation with ileitis findings in UC to distinguish these from ileal involvement in CD. In addition, several studies reported on the relation between the degree of inflammatory activity of the whole colon or cecum and the incidence of BWI[31,36,76]. In three (37.5%) of these studies[12,31,33], cecal inflammation was correlated with findings of BWI in the ileum. Jørgensen *et al*[33] found a significantly (P = 0.017) higher degree of cecal inflammation in patients with BWI. Interestingly, these three studies all reported BWI rates < 12% for UC in PSC, representing the lower limits of total rates found. While the high rates of right-sided and complete colonic involvement make an association between PSC-UC and BWI seem plausible, the overall percentage was found to be low. Available data on BWI are conflicting and studies differ in histological and diagnostic criteria. Our results emphasize the need to correlate histological findings in the ileum with cecal findings in order to accurately asses the presence of BWI in PSC-UC. Nonetheless, based on recent literature, BWI is more frequently observed PSC-UC compared with UC without PSC.

Similar to BWI, rectal sparing (RS) was reported as a common feature of IBD in PSC[11,30], together with extensive colitis and a predominance for right-sided inflammation[33]. Based on the reviewed papers, RS seems to be present in approximately one third of the patients with PSC-IBD, but large inconsistencies between studies are found. In most studies, the presence of RS was based on either endoscopic or histological data, but this approach has several limitations. Endoscopic imaging in quiescent disease, for example, could give the impression of macroscopic rectal sparing but does not exclude microscopic inflammation. Furthermore, a history of recent local steroid therapy can influence the endoscopic appearance of rectal mucosa, but only two studies[11,12] took this into account.

Finally, discrepancies in diagnosis of RS have been reported, showing rectal involvement in resected specimens despite frequently found rectal sparing in biopsies[37]. A comparison between rates of RS in PSC-IBD patients and rates found in IBD controls also yielded conflicting results. While all studies comparing RS incidence between PSC-IBD and IBD controls found higher rates in the former group, differences were significant in only two studies[11,25]. In contrast, Joo *et al*[31] found almost equal rates of RS in both UC and UC with associated PSC in their review of histological specimens. Both these results and the previously stated limitations regarding the diagnosis of RS, make it difficult to see RS as a distinct feature of the PSC-IBD phenotype. Diagnosis of true RS requires both endoscopic and histological absence of inflammation with the exclusion of recent (< 6 mo) local steroid therapy.

Based on limited results from small subgroup populations, we found that the characteristics of CD in PSC are similar to those found in UC associated with PSC. Disease activity is mild with an observed lower frequency of stricturing and penetrating disease compared with conventional CD. An isolated colitis is most common, being present in 55% of the patients with PSC-CD. Notably, the severity of PSC-CD may also be less, compared with PSC associated with UC. This was suggested by two studies[39,39,77] that found higher rates of small duct PSC in CD compared with PSC-UC, with one also finding a non-significant (P = 0.06) trend for increased survival[39].

PSC as a third and separate risk factor for CRC in concomitant IBD was first proposed by Broomé *et al*[78] in 1992 and was subsequently confirmed by several authors[22,38,46]. Other studies, however, have questioned this finding. They found that PSC did not constitute an additional risk of CRC[11,29,79] and proposed that PSC-IBD characteristics, *i.e.,* pancolitis and mild sub-clinical disease, facilitate the two previously mentioned risk factors for CRC development in IBD and explain the higher rates of CRC in PSC-IBD[11].

A comparison between the estimated risk of dysplasia and CRC development in PSC-IBD poses a challenge, as the use of statistical analyses and output parameters greatly varies between studies (Table 3). The most common reported format for colonic dysplasia and CRC development is the cumulative 10 year risk, which ranges from 0% to 11% for PSC-IBD. In all studies combined, development of dysplasia or CRC is observed in approximately 24% of patients with PSC and associated IBD. In contrast, in IBD controls the development of dysplasia or CRC is observed in 9% of the patients, with a reported estimated cumulative 10 years risk of 2%. In addition, a recent large French prospective study on neoplasia in IBD found an even lower rate of patients developing colonic neoplasia. In this study 0.3% of 19486 patients developed high grade dysplasia or CRC[80].

The included studies on both IBD and PSC-IBD differed greatly with respect to reported rates of dysplasia and CRC development. These differences are likely caused by the retrospective nature of the studies and by differences between centers regarding referral bias. Furthermore, discrepancies in the definition of dysplasia were found. Some studies reported all types of dysplasia, including low grade dysplasia, whereas other studies only registered colorectal cancer. While the progression of low grade dysplasia to advanced neoplasia in IBD has been shown to be low[81,82], it is worth noting that an increased risk of progression in PSC-IBD has been observed[83] making its inclusion justifiable. Based on these results and by taking study limitations into account, an increased risk of development of dysplasia and CRC in PSC-IBD can be confirmed. Future studies would benefit from a standard statistical output parameter to determine the risk of development of CRC in PSC-IBD.

Apart from an increased risk of CRC, a shorter interval between diagnosis of IBD and development of CRC in PSC-IBD was also reported[47]. The combined mean interval between development of IBD and colonic dysplasia or CRC found in patients with PSC-IBD is 19 years compared with 26 years for IBD patients without PSC. The observed median interval however, shows the opposite, with equal or even lower median intervals in IBD compared with PSC-IBD (Table 3). Although data on the age of both PSC-IBD patients and IBD patients without PSC at diagnosis of CRC were scarce, a lower median age at diagnosis of CRC was suggested for the former group. Based on these results, it is unclear whether the age of presentation and the interval between IBD diagnosis and development of large bowel neoplasia are lower in PSC-IBD compared with IBD. As both a younger age at CRC diagnosis and the efficacy of early surveillance in PSC-IBD have been confirmed and disputed[22,53], further studies are warranted.

As noted earlier, in PSC-IBD, a predisposition for development of right-sided colonic dysplasia and CRC has been observed[46,54,55,84]. While the reported percentages vary, a high predominance (> 60%) for dysplasia and CRC development in the proximal colon is confirmed by the majority of studies. The exact cause of increased dysplasia and CRC development in PSC-IBD however, remains unknown. A proposed mechanism is a cytotoxic and carcinogenic effect of secondary bile acids accumulating in the proximal colon due to defective small intestine reabsorption in cholestatic liver disease[85]. This assumption is strengthened by the association found between high concentrations of bile acids and the development of colon carcinoma in colitis[86].

The clinical course of IBD in PSC after liver transplantation was both observed to stabilize[87] or

to worsen[88]. In all studies combined, we found that approximately 32% of patients with PSC-IBD experience an exacerbation after OLT. For the development of *de novo* IBD after receiving a liver transplantation for PSC this was approximately 18%. These results are slightly lower than those presented in a recent review by Singh *et al*[89], who found IBD to worsen in a third of PSC-IBD patients after OLT. The different rates found for exacerbation and *de novo* presentation of IBD after liver transplantation for PSC have been contributed to the type of immunosuppressive medication, with lower rates reported for cyclosporin A and azathioprine compared with tacrolimus[59,88,90]. A proposed mechanism is the stronger suppression of interleukin-2 production by T-cells in tacrolimus compared to cyclosporin A, resulting in an inability to activate a regulatory response[59]. A subsequent study by the same group was not able to find a drug specific effect for tacrolimus on regulatory T cells in noninflamed colon mucosa of OLT patients[91]. Although further (pathophysiological) studies are required, the use of cyclosporin and azathioprine over tacrolimus seems to have a more favorable outcome on IBD after OLT for PSC, and should therefore be considered.

An early study by Cangemi *et al*[92] showed no beneficial effect of proctocolectomy on the progression or development of complications in PSC patients. In addition, careful consideration was urged in choosing proctocolectomy with ileostomy as the treatment of choice for colonic disease in PSC-UC, as it resulted in frequent development of parastomal varices. The safety and complications of proctocolectomy with ileal pouch-anal anastomosis (IPAA) in PSC-IBD were infrequently reported. While two out of three studies described proctocolectomy to be safe in PSC-IBD finding no increased mortality, a significant number of patients experienced postoperative morbidity (29% and 39% respectively[73,74]). Reported complications ranged from urinary tract and wound infections to bacterial cholangitis or hepatic decompensation. While peristomal varices are a common problem in ileostomy after proctocolectomy[93], none of the included studies demonstrated development of perianastomotic varices in IPAA. A less favorable outcome in PSC-IBD after colectomy was suggested, however, by a more recent study in which one-third of the patients progressed to OLT or death within an mean of 2.6 years[94]. In addition, this study stressed the importance of pre-operative assessment of liver function, finding a low preoperative platelet count and a low albumin level to be related to worse outcomes.

A frequently reported long term complication of IPAA in both IBD and PSC-IBD is pouchitis. Symptoms of pouchitis reported in the included studies are (bloody) diarrhea, abdominal cramping, urgency, malaise, and fever[95,96]. The definitions and diagnostic criteria for pouchitis however differed substantially between studies, making comparisons difficult. For instance, most authors found increased overall rates of pouchitis in PSC-UC, while Gorgun *et al*[71], who only looked at chronic pouchitis, found lower rates (13.8%) that were comparable to UC controls. Based on the included studies, we found that approximately 60% of PSC-UC patients experienced at least one episode of pouchitis after proctocolectomy with IPAA. This is more frequent when compared with conventional IBD after IPAA where pouchitis is observed in approximately one-third of the patients. Chronic pouchitis after IPAA is observed less frequently, but it still occurred more often in PSC-IBD when compared to IBD without PSC. Although pouchitis was found more common in PSC-IBD after IPAA, the rate of pouch failure does not seem to be increased and is comparable to IBD without PSC.

From a molecular perspective there is also increasing evidence that PSC-IBD is distinct from UC and CD. Large scale genetic studies performed within the International IBD Genetics Consortium have identified 163 independent genetic loci to be associated with IBD. The majority of these loci are shared between both UC and CD[97]. Similar studies have been performed within the International PSC study group, and they identified 16 independent loci outside the HLA region[98]. Mathematical modeling of these genetic variants showed that PSC is genetically more similar to UC than to CD which is comparable to the clinical observation. A striking finding of these studies is that only 50% of the PSC risk loci are shared with IBD. Based on power calculations and the fact that more than 70 % of the PSC patients had concomitant IBD one would have expected that many more of the 163 IBD loci would be associated with PSC than is currently observed[98,99]. These intriguing findings suggest that from a genetic perspective IBD-PSC is different from both CD and UC.

Based on our findings, several recommendations can be made concerning futures studies and the management of IBD associated with PSC.

Large differences between IBD rates in PSC were found. Therefore, to accurately assess or exclude the presence of IBD in PSC, a full colonoscopy with multiple biopsies from every segment and terminal ileum is required. Furthermore, the additional diagnostic criteria and a uniform definition are needed to assess the actual presence of backwash ileitis and rectal sparing in PSC-IBD patients.

Studies on the risk of the development of colonic neoplasia in PSC-IBD would benefit from a standard statistical outcome allowing comparison. The increase in the development of large bowel neoplasia in PSC-IBD patients observed in these studies requires frequent surveillance colonoscopy. The optimal timing of the start of surveillance warrants further study as recent data were found to be contradictory.

The use of cyclosporin A and azathioprine in-

stead of tacrolimus should be considered after liver transplantation for PSC, as the former were associated with a lower rate of IBD exacerbation and the development of *de novo* disease.

The safety and outcome of proctocolectomy in PSC-IBD depend upon the severity of liver disease, something which should always be assessed preoperatively. Ileal pouch-anal anastomosis reconstruction after colectomy in PSC-IBD was associated with a frequent development of pouchitis, and patients should be informed about this risk. Choosing for IPAA instead of ileostomy in PSC-IBD, however, seems favorable as the risk of development of local perianastomotic varices is low.

In conclusion, although IBD in PSC is common, there is a large variance in observed rates, which seems to be related to case ascertainment and, possibly, geographical variance. Based on the described clinical characteristics, the higher risk of development of colonic neoplasia, and the increasing molecular evidence, PSC-IBD should be regarded as a distinct phenotype of IBD, which differs from UC and CD and which therefore requires specialized management.

COMMENTS

Background

Inflammatory bowel disease (IBD) in Primary sclerosing cholangitis (PSC) often presents as quiescent disease affecting the whole colon. It is reported to be associated with backwash ileitis and rectal sparing. In addition an increased risk for the development of colorectal carcinoma has been described in several studies.

Research frontiers

Based on differences in disease characteristics it has been suggested that IBD in PSC represents a distinct phenotype in addition to ulcerative colitis (UC) and Crohn's disease. Studies looking at the characteristics for this phenotype however are limited.

Innovations and breakthroughs

While previous studies have summarized different aspects of IBD in PSC, studies providing a description of the PSC-IBD phenotype are limited. By reviewing existing literature on five relevant subthemes, an overview of the characteristics of the PSC-IBD phenotype is provided.

Applications

The results of this study suggest that IBD in PSC is different from conventional IBD and therefore represents a distinct phenotype which requires specialized management.

Terminology

PSC is a chronic cholestatic liver disease characterized by inflammation and fibrosis of both the intrahepatic and extrahepatic bile ducts. There is no curative therapy available and in most cases, PSC leads to liver cirrhosis and liver failure ultimately requiring liver transplantation. In the majority of cases PSC is accompanied by IBD most often UC.

Peer-review

The authors provided a systemic review on PSC-IBD characteristics compare to UC and IBD. The review is interesting and well done.

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SYSTEMATIC REVIEWS

Chronic hepatitis C genotype 1 treatment roadmap for resource constrained settings

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Abstract

AIM: To use existing hepatitis C virus (HCV) antiviral therapies as access to new treatments is limited.

METHODS: A PubMed search for randomised control trials or meta-analysis related to response-guided therapy of HCV genotype 1 patients was undertaken using pegylated interferon and ribavirin (PR), boceprevir (B) and telaprevir (T) and lead-in where response-guided therapy at TW4(TW4), 8(TW8), 10(TW10), or 12(TW12) based on HCVRNA(+) or HCVRNA(-). Studies presented at major conferences were also used. Where necessary, a post-hoc analysis was performed. A response-guided management roadmap was created based on sustained virological response (SVR).

RESULTS: Starting with PR, those with HCVRNA(-) at TW4 have > 86% SVR, while those are HCVRNA(+) have 34%-41.7% SVR. HCVRNA(-) TW4 patients can have 24 wk PR if HCVRNA < 400000 IU/mL. Alternatively, 28 wk BPR has similar SVR. If HCVRNA(+) at TW4, 72 wk PR leads to 53% SVR, hence BPR is a better option, and if HCVRNA(-) by TW8, 28 wk therapy is sufficient. If HCVRNA(+) at TW8, then HCVRNA should be checked at TW10 and TW12. By TW12, HCVRNA $\geqslant$ 100 IU/mL activates the stopping rule. This roadmap is applicable for treatment-naïve, treatment failures and cirrhotic patients. Validation from an Asia Pacific early access boceprevir program confirmed the findings that HCVRNA(-) at TW4, or TW8 conferred > 80% SVR, leading to the "80-80" rule.

CONCLUSION: Using a roadmap based on HCVRNA(-) at TW4 or TW8 (the "80-80" rule), high SVR can be achieved, and guide the best choices for treatment, and also reduces drug exposure in poor responders.

Key words: Chronic hepatitis C; hepatitis C virus RNA; Sustained virological response; Cirrhosis; Boceprevir; Telaprevir; Response-guided therapy; Peginterferon; Partial responder; Ribavirin

Core tip: Lex management of hepatitis C virus (HCV) genotype 1 using a simplified road map and "80-80" rule can help physicians manage their patients better. This roadmap distills the essential findings from using pegylated interferon and ribavirin as well as boceprevir

using treatment week 4 and 8 virological responses based on whether HCVRNA is detectable or not.

Lim SG. Chronic hepatitis C genotype 1 treatment roadmap for resource constrained settings. *World J Gastroenterol* 2015; 21(6): 1972-1981 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i6/1972.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i6.1972

INTRODUCTION

Chronic Hepatitis C is the one of most common causes of chronic viral liver disease worldwide afflicting 130-170 million persons[1], leading to significant morbidity and mortality due to liver disease and hepatocellular carcinoma of infected persons. The standard of care for hepatitis C therapy has long been pegylated interferon and ribavirin for 48 wk until 2011, but this therapy has been suboptimal in efficacy, safety and tolerance[2]. In 2011, the first generation protease inhibitors, boceprevir and telaprevir[3] were approved, then in 2013, simeprevir[4] and sofosbuvir[5] were approved by the United States Food and Drug Adminstration and then by the European Medicines Agency. They represent a substantial advance in efficacy with better tolerability, but come at a high cost. While resource rich countries can afford to use these treatments as first line therapy, access to such expensive, may be limited in resource constrained countries. Resource constrained countries are defined as those countries where the latest therapies sofosbuvir and simeprevir and not currently available nor likely in the near future, and where pegylated interferon and ribavirin (PR) ± boceprevir, are are still the standard of care. The roles of these new agents, in the context of PR with or without boceprevir and telaprevir is unclear in treatment naïve patients and in treatment failures, particularly where there is little experience on its use in resource constrained countries. The first generation protease inhibitors may still have a role in current management of chronic hepatitis C genotype 1 patients, although telaprevir is less widely available, and does not use a lead-in strategy. Hence, it is a somewhat less optimal therapeutic choice in a resource constrained setting. Alternatively, the complex treatment strategy for boceprevir based on presence of cirrhosis and previous treatment failure is compounded by the differences between the United States[6] and the European label[7] with regards to the duration of therapy. Different algorithms have been proposed for different types of hepatitis C virus (HCV) patients: treatment naïve, treatment experienced and for cirrhotics. Consequently, there is an important unmet need to simplify treatment strategy using a roadmap based management that can provide a practical guide for decision making at fixed timepoints (weeks 4, 8, 10 and 12) during HCV treatment. Undetectable or detectable HCVRNA was used as a decision tool as this only relied on the test result at that timepoint, in contrast to previous decision tools such as reduction of HCVRNA from baseline of or by stratifying the reduction in HCVRNA which requires at least two results.

MATERIALS AND METHODS

A PubMed search for meta-analyses or randomised control trials of hepatitis C genotype 1 treatment with PR for 24, 48 and 72 wk, where treatment week 4 (TW4) HCVRNA results and SVR were reported. Where no published articles were available, studies published in abstract format from key international liver meetings were utilised.

For boceprevir treatment, randomised control trials of phase 2 and 3 studies were used. In studies where the information on detectable or undetectable HCVRNA at TW4, 8, 10 or 12 were not presented, a post-hoc analysis was performed and data was extracted from the following studies: the phase 2 study (SPRINT-1) and the phase 3 (SPRINT-2 and RESPOND-2) boceprevir clinical trials[8-10], and PROVIDE studies[11]. Data was also included from analyses from a meta-analysis of cirrhotic patients[12] derived from five phase 3 boceprevir trials (SPRINT-2, RESPOND-2, peg2a, PROVIDE and anemia management study). The detailed methodology and primary outcomes from these studies have been are available in clinical trials.gov: NCT00423670, NCT00708500; NCT00705432, and NCT00910624. For telaprevir therapy, there is little information on lead-in and response-guided therapy.

End points and statistics

The primary end point in all studies was SVR, defined as undetectable HCVRNA 24 wk after completing treatment. Plasma HCVRNA levels were measured using COBAS TaqMan or COBAS TaqMan 2.0 (Roche Diagnostics) with respective lower limits of detection of 15 IU/mL and 9.3 IU/mL. Virologic response rates were assessed at various time points during therapy, at TW4, TW8, TW10 and TW12 after starting PR, and during boceprevir or telaprevir therapy.

RESULTS

Importance of TW4 HCVRNA as a predictor of SVR

TW4 has been validated as the most important predictor of SVR[13]. With regards to predictors of SVR[14]. multivariate analysis showed that the strongest predictor of SVR in Caucasians and Black patients given boceprevir triple therapy was $\geqslant$ 1 log reduction in HCVRNA, even stronger that the IL28B cc genotype. In the largest Asian study of peginterferon and ribavirin[15], multivariate analysis again showed that week 4 undetectable HCVRNA was a stronger predictor

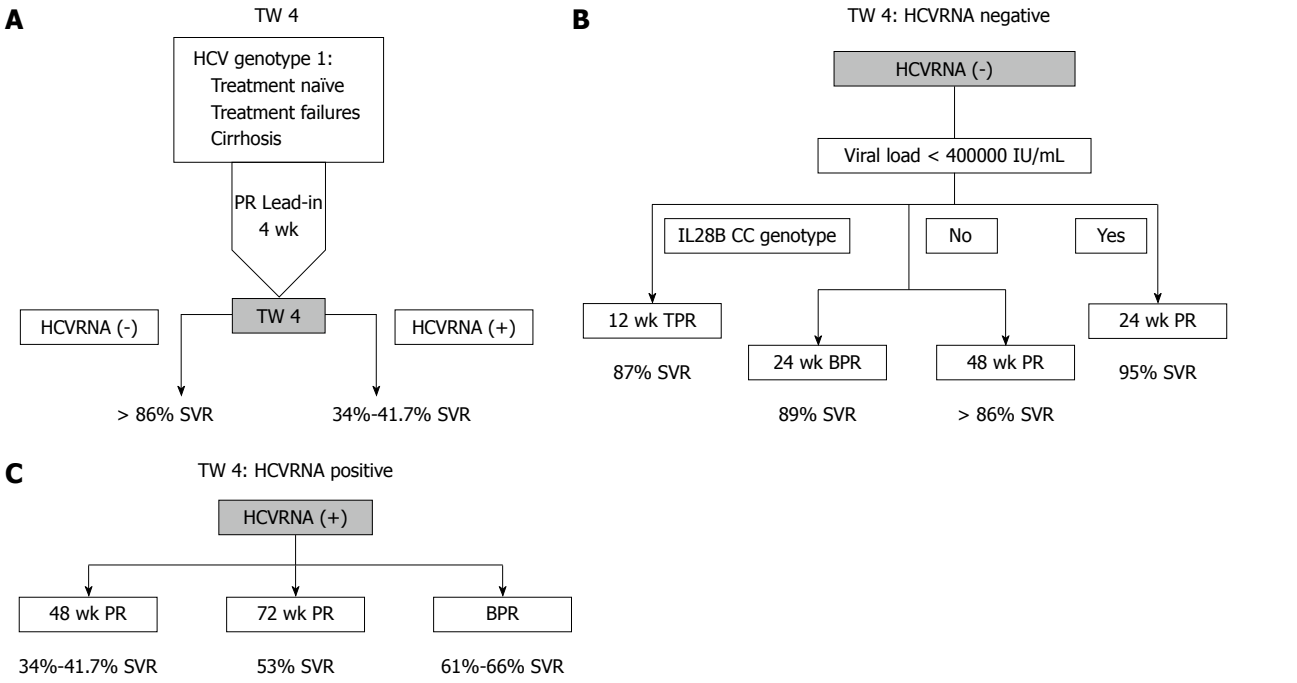


Figure 1 Treatment week 4 (A), hepatitis C virus RNA negative (B) and hepatitis C virus RNA positive (C). A: All patients eligible for PR therapy regardless of whether they are treatment naïve, previous treatment failures or cirrhotics. Patients who achieve RVR after 4 wk lead-in either have detectable or undetectable HCVRNA. Those with undetectable HCVRNA have SVR > 86% while those with detectable HCVRNA have SVR 34%-41.7%; B: These patients have a high possibility of SVR so PR therapy can be shortened to 24 wk if they have baseline viral load < 400000 IU/mL). If they do not have good baseline predictors, therapy can also be shortened by addition of boceprevir (4 wk lead-in, 24 wk boceprevir + PR). If they also have IL28B CC genotype, telaprevir and PR can be used for 12 wk as well. Alternatively, they can continue on to 48 wk PR; C: These patients have a low possibility of SVR (41.7%) hence the alternative is to extend PR to 72 wk (53% SVR) or to add boceprevir (61%-66% SVR). TW: Treatment week; HCV: Hepatitis C virus; PR: Pegylated interferon and ribavirin; SVR: Sustained virological response.

of SVR than IL28B cc genotype. Consequently while IL28B genotype is still an important predictor, it may not be crucial in the decision to start therapy since week 4 HCVRNA is the stronger predictor.

SVR and TW4 response

The largest randomized control trial (RCT) of 48 wk PR[13] had few Asians, but the SVR rate in those with undetectable HCVRNA at TW4 was 86% while those with detectable HCVRNA had SVR of 33.9%. In the largest Asian RCT of 48 wk PR[15], those with undetectable HCVRNA at TW4 had 98% SVR, but if HCVRNA was detectable, SVR was 34.5%, remarkably similar to non-Asians. Consequently the first decision point of the roadmap is TW4 (Figure 1A). The difference in the two studies was that undetectable HCVRNA at TW4 was achieved in only 8.9% of patients in the IDEAL study, but 54.8% in the Asian study due to the high prevalence of the IL28B good response genotype[16].

TW4 Response: Those who have undetectable HCVRNA

With a high SVR of 86%-98% with 48 wk PR, can therapy be shortened? A meta-analysis[17] showed that SVR was significantly higher with 48 wk PR, 94.1% *vs* 79.7% for 24 wk PR (RR = 1.15; 95%CI: 1.07-1.24; $P < 0.0001$). But those with low baseline viral load (< 40000 IU/mL), had no difference in SVR, 95.5% for 48 wk PR *vs* 90.6% in the 24 wk PR (RR = 1.05; 95%CI: 0.99-1.11; P = not significant). Consequently, only those low baseline viral load can have 24 wk PR.

Can BPR for 24 wk be an alternative? In the SPRINT-2 study[9], those who achieved undetectable HCVRNA at TW4 and TW8 had 24 wk BPR had 88% SVR, compared to 97% SVR of 97% in those who had 48 wk BPR (P = NS), making this an alternative to 48 wk PR (Figure 1B).

An alternative to boceprevir is telaprevir. In the CONCISE study[18], treatment naïve or relapser patients with the IL28B CC genotype who had undetectable HCVRNA at TW4 who were randomised to 12 wk of telaprevir and PR (TPR) achieved 87% SVR compared to 97% in the 12 wk TPR+12 wk PR group (P = NS). This is the only study telaprevir was used with lead-in based on undetectable HCVRNA at TW4.

Those who have detectable HCVRNA at TW4

A Cochrane systematic review[19] addressed whether 72 wk PR was superior to 48 wk PR in these patients. The meta-analysis showed 41.7% SVR for 48 wk PR, with a risk ratio of 1.27 (95%CI: 1.07-1.50), or 53% (95%CI: 44.6%-62.6%) SVR for 72 wk PR (Figure 1C). Importantly, this meta-analysis included both Caucasian and Asian studies with no significant difference between them in tests of heterogeneity.

The SVR rate of patients who have detectable HCVRNA at TW4 in the SPRINT-2 study[9], was 65%

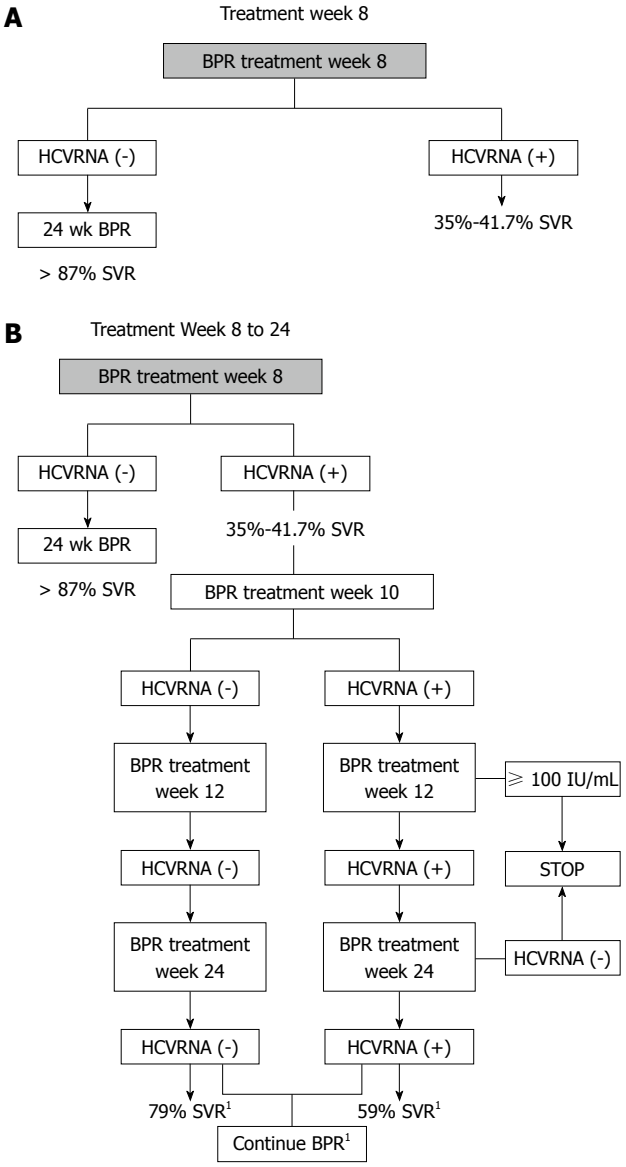


Figure 2 Treatment week 8 (A) and treatment week 8 to week 24 (B). A: These patients regardless of the treatment week 4 responses have a high possibility of SVR if they are HCVRNA negative (SVR > 87%) and are eligible for shortened therapy but if they are HCVRNA positive, then SVR rates are low, 35%-41.7%; B: After addition of boceprevir, with undetectable HCVRNA, these patients are eligible for shortened therapy (4 wk lead-in, 24 wk boceprevir +PR). However if they are HCVRNA positive then HCVRNA at weeks 10 and 12 are useful to guide therapy. Those who are HCVRNA ⩾ 100 IU/mL at week 12 or have detectable HCVRNA at week 24 fulfill the stopping rules. [1]Not eligible for shortened therapy - should complete 32 wk BPR ± 12 wk PR or complete 44 wk BPR. HCV: Hepatitis C virus; PR: Pegylated interferon and ribavirin; SVR: Sustained virological response.

in the RGT group and 66% in 48 wk fixed duration therapy group, while in RESPOND-2, no analysis was performed of SVR rates in those with detectable HCVRNA at TW4, as the analysis was based on interferon responsiveness, defined as HCVRNA decline at TW4 by < 1log$_{10}$ ⩾. The PROVIDE study[11] was analysed in a similar manner. Fortunately, a post hoc analysis can be performed using the abstract presented by Vierling *et al*[20] which showed the different levels of interferon responsiveness at TW4 for RESPOND-2. SVR in those with detectable HCVRNA at TW4 was calculated to be 61% (95/156) (range: 31%-90%) in the RGT group, and 66% (103/156) (range: 13%-100%) for the 48 wk fixed duration group. Consequently, the SVR rate in those who have detectable HCVRNA at TW4 is remarkably similar, 61%-66% regardless whether patients are treatment naïve or failures, and regardless of RGT or 48 wk fixed duration therapy (Figure 1C). However, SVR could be as low as 33% if the patient was a "null responder" or as high as 80% in "partial responders" at TW4[21].

TW8 response

HCVRNA undetectable with BPR: In SPRINT-2[9], treatment naïve patients with undetectable HCVRNA at TW8, had 88% SVR with RGT compared to 90% SVR with BPR 48 wk. In the RESPOND-2 study[10], prior treatment failure patients with undetectable HCVRNA at TW8, had 86% SVR with RGT compared to 88% SVR with 48 wk BPR. These findings confirm that those with undetectable HCVRNA will benefit from 24 wk BPR (Figure 2A). Even treatment naïve poor interferon responders at TW4, or "null responders" (< 1log HCVRNA reduction at TW4), in a post-hoc analysis of the SPRINT-2 and RESPOND-2 studies, who have undetectable HCVRNA at TW8, achieve 83% SVR[22].

HCVRNA detectable with BPR: In the SPRINT-2[9], treatment naïve late responders (HCVRNA detectable at TW8 but undetectable at treatment week 24) achieved 73.1% SVR with RGT compared to 75% SVR with 48 wk fixed duration therapy. However, when all patients with detectable HCVRNA at TW8 are examined in Th SPRINT-2 study, the SVR rate is only 36% for RGT and 40% for 48 wk fixed dose therapy, as these include patients who have detectable HCVRNA at TW24 (which invoke the futility rule for stopping therapy). However, we do not need to wait to TW24, as by TW12 the futility rule can also be applied to patients with HCVRNA ⩾ 100 IU/mL[23] (Figure 2B). In the RESPOND-2 study[10], SVR data on those with detectable HCVRNA at TW8 were not presented but can be extracted as a post-hoc analysis. Since the total SVR rates were known for group 2 (95/162, 58.6%) and group 3 (107/161, 66.4%) and the rates for patients with undetectable HCVRNA at TW8 are known, for group 2 (74/161, 46%), and group 3 (84/161, 52%), as well as SVR rates are known for group 2 (64/74, 86%), and group 3 (74/84, 88%), we can calculate SVR rates in those with detectable HCVRNA at TW8 in group 2 (31/88, 35%), and group 3 (33/80, 41.2%). Group 2 patients were in the RGT arm, and group 3 were in the fixed duration 48 wk therapy arm. Pooling group 2 and 3 together we can calculate that 168/323 (52%) of prior treatment failure patients had

detectable HCVRNA at TW8.

Consequently, both SPRINT-2 and RESPOND-2 studies demonstrate remarkably similar results for those with detectable HCVRNA at TW8, with SVR rates of 35%-36% for RCT and 40%-41.2% for fixed duration 48 wk therapy, regardless of their prior response to therapy.

TW10 and 12 response

For patients who have detectable HCVRNA at TW8, rather than wait for TW12, undetectable HCVRNA at TW10 can also be used to determine if SVR is likely (Figure 2B). In an post-hoc analysis combining SPRINT-2 and RESPOND-2 studies[24], those with detectable HCVRNA at TW8 were evaluated at TW10 and undetectable HCVRNA at TW10 and at TW12 conferred SVR of 79% while a detectable HCVRNA at TW10 followed by undetectable HCVRNA at TW12 conferred a SVR of 59% (Figure 2B), provided they maintained undetectable HCVRNA through to treatment week 24.

Treatment naïve vs treatment failure patients

Based on the data above, it would seem that response-guided management using undetectable HCVRNA successively at TW4, 8, 10 and 12 predict a high chance of SVR regardless of whether a patient is treatment naïve or a prior treatment failure, but does this apply to cirrhotics?

Cirrhosis

It is well known that SVR is impacted by the presence of progressive fibrosis and cirrhosis. Subanalyses from randomised control trials show that RVR is lower in cirrhotic patients compared to non-cirrhotics[25,26]. In the CHARIOT study[25], RVR in F0-2 patients was 24% compared to 18% in those with F3-4. Overall SVR was much lower in those with cirrhosis (10%) compared to those without fibrosis, F0 (70%). Those with RVR and F0-2 achieved 80% SVR but those with F3-4 had only 63% SVR. Bruno *et al*[26], collated three RCTs showing that RVR in those without advanced fibrosis was 23.6% compared to 11% with advanced fibrosis. SVR was lower in those with cirrhosis (33%) compared to those without bridging fibrosis (60%). Notably, those who achieved RVR without advanced fibrosis had SVR of 95% compared to 89% in those with advanced fibrosis.

Consequently, responses to boceprevir in cirrhotics needs evaluation. A meta-analysis of all boceprevir treated patients with cirrhosis[12] found that all those with undetectable HCVRNA at TW8 has similar SVR, 86% for F0-2, 85% for F3 and 89% for F4. Undetectable HCVRNA at TW8 was the strongest predictor of SVR by multivariate analysis with OR = 10.57 (95%CI: 5.23-21.36). Consequently, we can be confident that the findings from non-cirrhotics are similar in cirrhotics (Figure 2A).

Duration of boceprevir triple therapy

Based on the response-guided management proposed for boceprevir (Figures 2B and 3), patients who have undetectable HCVRNA at TW8 can shorten therapy to 24 wk of BPR (after lead-in). This is true even for patients with cirrhosis and even for those who had previous PR treatment failure. However, it is important to note that the boceprevir label[23] advises duration of boceprevir triple therapy based on whether the patient is treatment naïve, is an early or late responder, has prior treatment failure or has cirrhosis. Only in treatment naïve early responders is it recommended to for 24 wk BPR. In cirrhosis and previous null responders, it is recommended to have 44 wk of BPR (after 4 wk lead-in). For the remainder, late responders, prior treatment failures the recommendation is for 32 wk of BPR (after 4 wk lead-in) with or without an extra 12 wk PR tail. We should be mindful that the 32 wk BPR proposal is not supported by evidence, only by modeling. This confusing recommendation leaves much to be desired since it does not simplify management, and that the prior documentation of null response can be rather poor, not to mention that diagnosis of cirrhosis with non-invasive markers is not always optimal, making it difficult at times to select the correct duration of therapy. Although our proposed roadmap simplifies therapy considerably in those who are good responders regardless of prior treatment failure or presence of cirrhosis, when in doubt, the package insert should be followed[23].

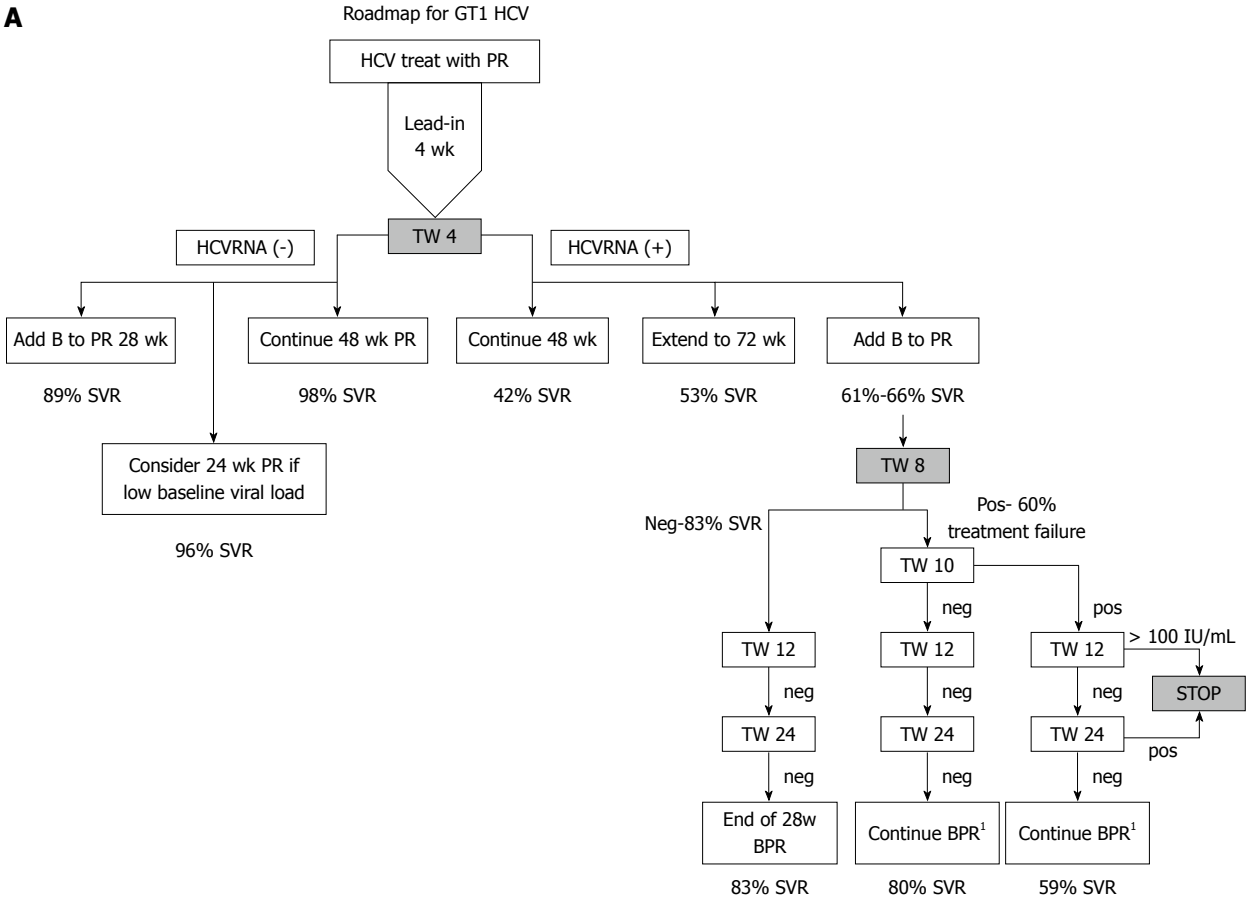
How long should those with detectable HCVRNA at TW8 be treated with BPR? Based on the package insert, at least 32 wk of BPR ± 12 wk of PR tail. Given these patients are the most likely to fail therapy, they should be given the maximum benefit with 44 wk BPR, as supported by the SPRINT-2 and RESPOND-2 studies.

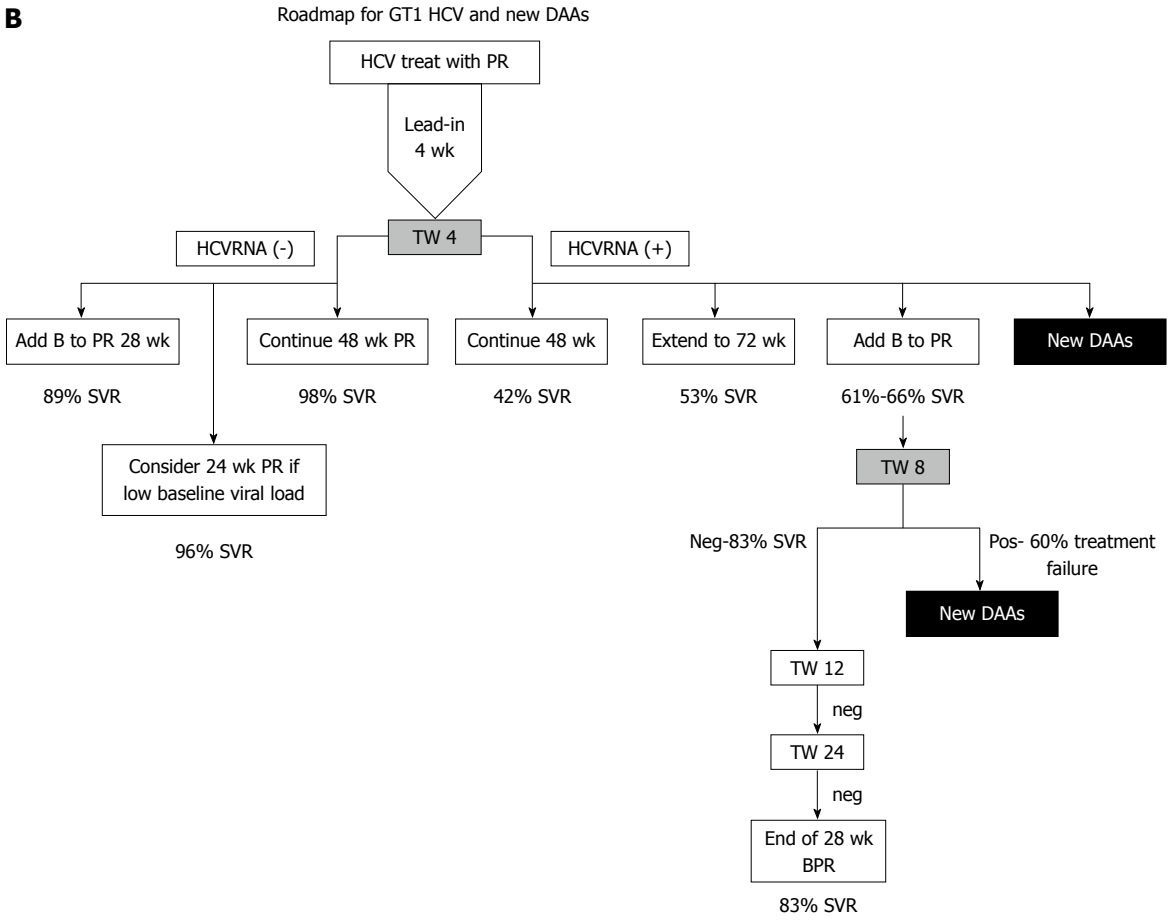
Consolidated roadmap based management

In Figure 3A, a consolidated management approach to HCV genotype 1 is proposed regardless of the baseline characteristics of treatment naïve, treatment failure or cirrhosis.

80-80 Rule

Using the roadmap based management, we can invoke an "80-80" rule, that is, if undetectable HCVRNA is achieved at TW4, > 80% SVR will be obtained, or using boceprevir, undetectable HCVRNA is achieved at TW8, > 80% SVR will also be obtained (Figure 3C). The possibility of failure if HCVRNA is detectable at TW8 is higher. In a post-hoc analysis from SPRINT-2, there were 64% (83/129) failures in the RGT arm, and 60% (79/131) in the fixed duration


A
Roadmap for GT1 HCV
HCV treat with PR
Lead-in 4 wk
TW 4
HCVRNA (-)
HCVRNA (+)
Add B to PR 28 wk
Continue 48 wk PR
Continue 48 wk
Extend to 72 wk
Add B to PR
89% SVR
98% SVR
42% SVR
53% SVR
61%-66% SVR
Consider 24 wk PR if low baseline viral load
96% SVR
TW 8
Neg-83% SVR
Pos- 60% treatment failure
TW 10
neg
pos
TW 12
TW 12
TW 12
> 100 IU/mL
STOP
neg
neg
neg
TW 24
TW 24
TW 24
pos
neg
neg
neg
End of 28w BPR
Continue BPR1
Continue BPR1
83% SVR
80% SVR
59% SVR


B
Roadmap for GT1 HCV and new DAAs
HCV treat with PR
Lead-in 4 wk
TW 4
HCVRNA (-)
HCVRNA (+)
Add B to PR 28 wk
Continue 48 wk PR
Continue 48 wk
Extend to 72 wk
Add B to PR
New DAAs
89% SVR
98% SVR
42% SVR
53% SVR
61%-66% SVR
Consider 24 wk PR if low baseline viral load
96% SVR
TW 8
Neg-83% SVR
Pos- 60% treatment failure
New DAAs
TW 12
neg
TW 24
neg
End of 28 wk BPR
83% SVR

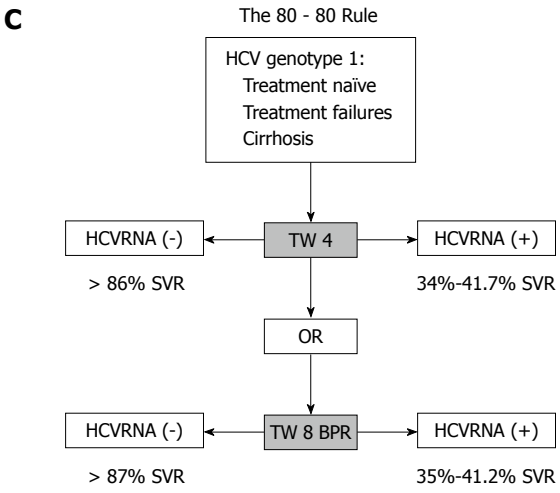


Figure 3 Roadmap for GT1 hepatitis C virus (A), Roadmap for GT1 hepatitis C virus and new direct acting antivirals (B) and the "80-80" Rule (C). A: Full roadmap showing timepoints, predicted SVR based on undetectable HCVRNA at timepoints, and alternative choices at each timepoint. [1]Not eligible for shortened therapy - should complete 32 wk BPR ± 12 wk PR or complete 44 wk BPR; B: Modification of the roadmap showing that new DAAs can be considered at Treatment week (TW)4 or TW8 if HCVRNA is positive; C: Main points of the roadmap showing that if HCVRNA is negative at TW4 (on PR) or 8 (on BPR) then there is > 80% SVR. If HCVRNA is positive at week 4, then there is 41.7% SVR if 48 wk PR is continued, but at week 8, if HCVRNA is positive while on BPR then the possibility of SVR is 35%-41.2%. HCV: Hepatitis C virus; PR: Pegylated interferon and ribavirin; SVR: Sustained virological response.

arm, while in RESPOND-2, failures in the RGT arm were 64.7% (57/88), and the fixed duration arm was 58.8% (47/80). Overall, with detectable HCVRNA at TW8, the chance of failure is approximately 60%.

Validation of roadmap

The proposed roadmap was derived from randomised control trials but how would it perform in real life? In an early access program for boceprevir, the Boceprevir Named Patient Program (BNPP), investigators from Singapore, Thailand, Malaysia and Australia decided to pool their patients into a study, Boceprevir Early-Access For Advanced Fibrosis/Cirrhosis To Evaluate Outcomes In Asia-Pacific HCV Genotype 1 Non-Responders And Relapser Patients (BEACON study). A total of 150 patients (Asians = 86, Caucasians = 63) were enrolled and these patients had to have previous treatment failure with PR, and had to have advanced fibrosis or cirrhosis, but no decompensated liver disease. The final data analysis is currently being prepared for publication (manuscript in press, World J Gastroenterology). Applying the roadmap strategy to the BEACON study, those who have undetectable HCVRNA at TW4, have 100% SVR (14/14), while those who have undetectable HCVRNA at TW8 have 87% SVR (74/85). Those who have detectable HCVRNA at TW4 have 58% SVR (72/124) and those who have detectable HCVRNA at TW8 have 22% SVR. These patients represent the most difficult to treat subtypes, as they have had previous treatment failure, and also have advanced fibrosis or cirrhosis. The findings of BEACON confirm that in real-life settings, the roadmap strategy is indeed valid.

When to introduce second generation DAAs

When the "80-80" rule is not met, treatment failure becomes more likely. This rule can be met as early as TW4, when an alternative is to introduce new DAAs (Figure 3B) such as sofosbuvir and PR, however, there is no data on the SVR rates in treatment-experienced patients. An alternative is simeprevir and PR, but in null responders, SVR was 58.8%[27]. As patients who are HCVRNA positive at TW4 are enriched for null responders, simeprevir and PR would not be an optimal alternative. At TW8, detectable HCVRNA also increases the likelihood of treatment failure to 60% making alternative new DAAs an option (Figure 3B). Again such patients would be enriched for null responders hence simeprevir and PR is a suboptimal, nor is there data on such patients treated with sofosbuvir and PR. However, such patients can be rescued in the near future with the new generation of interferon-free oral treatments, and null responders achieve > 90% SVR with the Abbvie quad regimen[28], the sofosbuvir-ledispavir ± ribavirin combination[29], and the sofosbuvir-simeprevir combination[30]. Sofosbuvir and ledispavir ± ribavirin was also effective in patients with baseline protease inhibitor resistance associated variants[31].

DISCUSSION

These are rather exciting times for patients who suffer from chronic hepatitis C. Sofosbuvir[5] and simeprevir[4] have both been very recently been approved for treatment of chronic hepatitis C which appear to be a substantial improvement over the current DAAs, telaprevir and boceprevir. However, access to the newly approved DAAs will be gradual as approval in different countries and regions are likely to take years. In the interim, hepatitis C treatment in each country or region needs to adapt to the evolving

situation and to determine the best strategy for SVR, bearing in mind cost of therapy is a critical factor in resource constrained settings. In the immediate and near term, PR is still likely to be the mainstay of therapy in resource constrained settings since not only is it the only available therapy, but even boceprevir and telaprevir constitute a considerable cost, not taking into consideration adverse events, tolerability and toxicity.

The current analysis is a distillation of existing literature showing that by using TW4 and TW8 HCVRNA, dichotomised to detectable or undetectable, provides a high level of predictability for SVR, simplifying the complexity and confusion of using log reduction in HCVRNA, and stratifying by type of prior treatment failure or presence of absence of cirrhosis. This response-guided approach is particularly appealing in countries where IL28B CC genotype is prevalent, since a greater proportion of patients have undetectable HCVRNA at TW4. In countries where the IL28B T genotype is more prevalent, the TW4 response is likely to be poor, and more reliance will be placed on the TW8 response. At TW4 or TW8, if HCVRNA is undetectable, SVR > 80%, leading to the "80-80" rule, making this reassuring for both physicians and patients that SVR is likely, encouraging compliance, and making tolerability of adverse events more bearable. Moreover, the roadmap allows patients to terminate treatment as early as TW12 if HCVRNA ⩾ 100 IU/mL, if response to therapy is unfavorable, based on the stopping rule. Consequently the roadmap also can provide at each timepoint the likelihood of treatment failure and such patients can have the option of stopping therapy and consider the new generation of DAAs, where data is available.

There are some caveats to the roadmap strategy since it provides likelihood of SVR in those who are able to tolerate therapy. A significant proportion of patients have adverse events to pegylated interferon, ribavirin and boceprevir or the combination, and discontinuation of therapy occurs in a substantial proportion of patients due to adverse events. In the CUPIC study[32] patients who were at risk of developing severe complications including sepsis and death, had a low serum albumin < 35 g/dL and a low platelet count < 100000/L. Consequently boceprevir or telaprevir therapy is not recommended in such patients due to the high risk of these complications. In such patients, they should look to interferon-free therapy and the new generation of DAAs.

With the new generation of DAAs around the corner, the era of interferon-free regimens is around the corner. Moreover, approval of the new DAAs may take as long as 3-4 years in some Asian countries. Consequently the new DAAs, which currently come with a high cost of therapy, having cheaper but almost as effective alternatives a choice to be considered. Consequently, PR is still likely to be a first line therapy for cost reasons, both in countries with and without reimbursement. One can speculate that the current first generation DAAs could be used as second-line therapy when RVR fails, and the new DAAs could become a third line therapy when both first and second line are ineffective. Clearly this can change if cost structures alter. In the ideal world, interferon based regimes are inconvenient and carry adverse events which makes tolerance and compliance an important issue. While the future points to an interferon-free HCV treatment, we should discount the utility of interferon based regimens as an interim measure for patient management.

COMMENTS

Background

Although new all oral antiviral agents for hepatitis C virus (HCV) are available in some countries, many countries who have constrained resources still are using pegylated interferon and ribavirin and first generation direct-acting antivirals.

Research frontiers

There is still confusion on how to use pegylated interferon, ribavirin with or without first generation direct-acting antivirals, and a distillation of the available best evidence can provide a practical guide for on-treatment management.

Innovations and breakthroughs

On-treatment response based on detectable or undetectable HCVRNA at week 4 or 8 can guide continuing or changing treatment. This can be summarised as the "80-80" rule which means SVR ⩾ 80% can be achieved if HCVRNA is negative at week 4 or 8 of therapy.

Applications

This roadmap is applicable to all HCV genotype 1 patients regardless of whether.

Peer-review

Dr. Lim SG described the road map for antiviral therapy to HCV genotype 1 patients. This study is review article. As the author pointed out, direct acting antivirals present substantial advance in efficacy with better tolerability, though they are very expensive.

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CASE REPORT

Successful treatment of conversion chemotherapy for initially unresectable synchronous colorectal liver metastasis

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Abstract

A 72-year-old woman with a sigmoid colon cancer and a synchronous colorectal liver metastasis (CRLM), which involved the right hepatic vein (RHV) and the inferior vena cava (IVC), was referred to our hospital. The metastatic lesion was diagnosed as initially unresectable because of its invasion into the confluence of the RHV and IVC. After she had undergone laparoscopic sigmoidectomy for the original tumor, she consequently had 3 courses of modified 5-fluorouracil, leucovorin, and oxaliplatin (mFOLFOX6) plus cetuximab. Computed tomography revealed a partial response, and the confluence of the RHV and IVC got free from cancer invasion. After 3 additional courses of mFOLFOX6 plus cetuximab, preoperative percutaneous transhepatic portal vein embolization (PTPE) was performed to secure the future remnant liver volume. Finally, a right hemihepatectomy was performed. The postoperative course was uneventful. The patient was discharged from the hospital on postoperative day 13. She had neither local recurrence nor distant metastasis 18 mo after the last surgical intervention. This multidisciplinary strategy, consisting of conversion chemotherapy using FOLFOX plus cetuximab and PTPE, could contribute in facilitating curative hepatic resection for initially unresectable CRLM.

Key words: Initially unresectable; Colorectal liver metastasis; Conversion chemotherapy; Cetuximab; Percutaneous transhepatic portal vein embolization

Core tip: A 72-year-old woman with a sigmoid colon

cancer and a synchronous colorectal liver metastasis (CRLM) was referred to our hospital. The metastatic lesion was diagnosed to be initially unresectable. After she had undergone laparoscopic sigmoidectomy for the original tumor, she consequently had 6 courses of modified 5-fluorouracil, leucovorin, and oxaliplatin plus cetuximab, resulting in conversion chemotherapy. Preoperative percutaneous transhepatic portal vein embolization was performed to secure the future remnant liver volume. Finally, a right hemihepatectomy was successfully performed. The postoperative course was uneventful. She had no recurrence for 18 mo. This multidisciplinary strategy could contribute in facilitating curative hepatic resection for initially unresectable CRLM.

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INTRODUCTION

The incidence of colorectal cancer is increasing, and it is now the fourth leading cause of cancer deaths worldwide[1]. According to GLOBOCAN 2008 estimates, about 12.7 million cancer cases and 7.6 million cancer deaths were estimated to have occurred in 2008[2]. Over half of patients with colorectal cancer will develop metastatic disease, with a quarter having distant metastatic lesions at diagnosis, often in the liver[3]. Although hepatic resection remains the only potentially curative treatment in patients with colorectal liver metastasis (CRLM)[4-7], only 15% to 20% of patients with CRLM are suitable for surgical resection[8,9]. Here, we report a case of conversion chemotherapy using 5-fluorouracil, leucovorin, and oxaliplatin (FOLFOX) plus cetuximab, combined with portal vein embolization, which led to a successfully curative liver resection in a patient with initially unresectable synchronous CRLM.

CASE REPORT

A 72-year-old woman with a high level of carcinoembryonic antigen (CEA) was referred to our hospital for the further diagnosis from a previous physician. She had no previous history of serious diseases, operations, or hospitalizations. She had a family history of gastric cancer on her mother's side. Laboratory workup showed that CEA level had increased to 27.0 ng/mL, and serum levels of transaminases were slightly elevated. Total colonoscopy revealed a tumor at the sigmoid colon. Computed tomography (CT) revealed a synchronous CRLM. It was diagnosed as unresectable due to its invasion of the right hepatic vein (RHV) and the inferior vena cava (IVC) (Figure 1A and B). Thus, she underwent just a sigmoidectomy with lymph node dissection. Histopathological analysis resulted in the diagnosis of a well-to-moderately differentiated adenocarcinoma. Since the cancer cells were found to have wild-type KRAS, a combination therapy of mFOLFOX6 with cetuximab [Day 1: 5-fluorouracil (5-FU) 400 mg/m^2 bolus injection; leucovorin (LV) 200 mg/(m^2.2 h) with oxaliplatin (L-OHP) 85 mg/(m^2.2 h); cetuximab 250 mg/(m^2.2 h); 5-FU 2400 mg/(m^2.46 h) continuous infusion; Day 8: cetuximab 250 mg/(m^2.1 h), every 2 wk] was chosen as the first-line chemotherapy, considering the possibility of conversion chemotherapy. Follow-up CT revealed that the CRLM had a partial response, and that the confluence of the RHV and IVC was free from cancer invasion after 3 courses of systemic chemotherapy (Figure 1C and D). In addition, the serum level of CEA decreased significantly (Figure 2). The residual liver volume was regarded as insufficient for right hemihepatectomy. Thus, percutaneous transhepatic portal embolization (PTPE) was carried out after an additional 3 courses of chemotherapy. Three weeks after PTPE, CT revealed an increase of the estimated future remnant liver ratio from 36.2% to 46.9%, with no detectable presence of any other metastatic lesion. This CRLM was finally regarded as resectable with a normal hepatic functional reserve (Table 1). Six weeks after the last course of chemotherapy, a right hemihepatectomy and cholecystectomy were performed because of the invasion to the right branch of the portal vein. After the mobilization of the right lobe and the completion of hepatic transection, the RHV and a part of the IVC was side-clamped with a Satinsky clamp and divided. The surgical margins, observed with a frozen section, had no malignancy. The side-clamped IVC was simply closed with a continuous suture without severe stenosis. The postoperative course was uneventful. The patient was discharged on postoperative day 13. The histopathological analysis revealed a well-differentiated adenocarcinoma, consistent with CRLM. The tumor was comprised of approximately 50% viable cancer cells, and the remainder was necrotic (Figure 3). The patient had an additional 6 courses of mFOLFOX6 after hepatectomy. She had neither local recurrence nor distant metastasis 18 mo after the last surgical intervention.

DISCUSSION

Liver resection is the only potentially curative treatment with an expectation of long-term survival in patients with CRLM[4-7]. However, approximately 80% of patients with CRLM have unresectable disease,

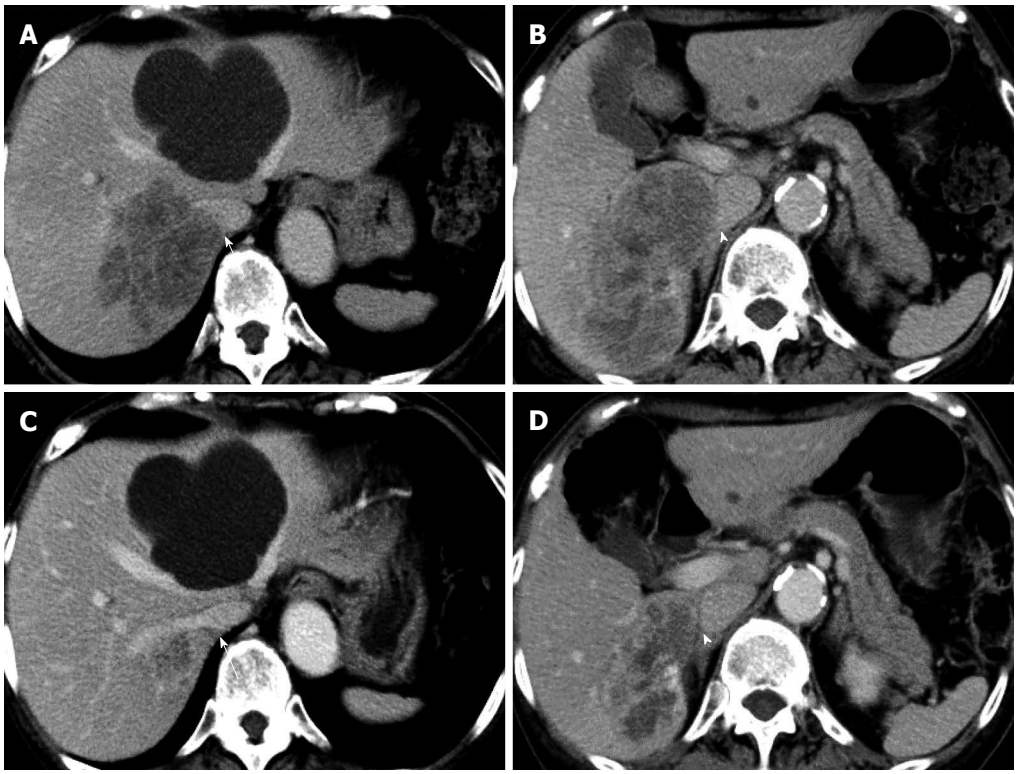

Figure 1 Enhanced computed tomography. A, B: Before chemotherapy. A huge synchronous colorectal liver metastasis was involving the right hepatic vein (RHV; arrow) and the inferior vena cava (IVC; arrowhead); C, D: After chemotherapy. The tumor was dramatically reduced, and the IVC was isolated from the tumor (arrowhead).

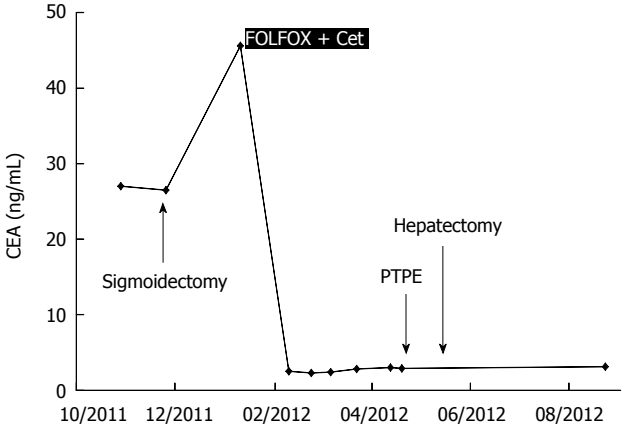

Figure 2 Serum levels of carcinoembryonic antigen. FOLFOX: 5-fluorouracil, leucovorin, and oxaliplatin; Cet: Cetuximab; PTPE: Percutaneous transhepatic portal vein embolization.

and long-term survival is poor in this setting[10]. In selected patients with unresectable metastases, CRLM may be down-staged by systemic chemotherapy with or without molecular targeted therapy, so that liver resection may be completed[11-15]. Once a complete curative resection is achieved, long-term survival would be expected, even in patients with initially unresectable CRLM[10,16-19]. Moreover, there is a positive correlation between the response rate to chemotherapy and the resection rate of liver metastases[20]. Therefore, response rates are very important when selecting patients for resection.

Currently, L-OHP and irinotecan have been widely used for patients with CRLM. The resection rate is significantly higher in patients receiving FOLFOX (5-FU + LV + L-OHP) than in those receiving FOLFIRI (5-FU + LV + irinotecan), according to a randomized GERCOR study[21]. To make matters worse, preoperative treatment with irinotecan has been reported to be associated with an increased risk of steatohepatitis. Steatohepatitis is associated with an increase in 90-day mortality after hepatic surgery[22]. While, L-OHP-based chemotherapy is associated with a significantly higher incidence of sinusoidal obstruction syndrome (SOS)[23,24]. SOS resulted in a poorer hepatic functional reserve and in a higher complication rate after major hepatectomy[24], but with no increase in mortality[22]. Therefore, we selected mFOLFOX6 as first-line chemotherapy, considering the future possibility of liver surgery.

The development of efficient molecular-targeted drugs, such as cetuximab or bevacizumab, have opened new perspectives in the treatment of resectable and unresectable liver metastases. In patients with KRAS wild-type tumors, chemotherapy with cetuximab yields high response rates compared with historical controls, and leads to significantly increased resectability[25-29]. The phase Ⅲ NORDIC7 and COIN trials reported that first-line L-OHP-based chemotherapy plus cetuximab has no confirmed

Table 1 Laboratory data

WBC	6000/L
RBC	343 × 10^4/L
Hb	10.7 g/dL
Hct	34.6%
Plt	24.8 × 10^4/L
PT	100.1%
HPT	75.7%
AT3	107%
CEA	2.9 ng/mL
CA19-9	14.1 U/mL
ICG-R15	6.7%
CRP	0.077 mg/dL
T-Bil	0.5 mg/dL
AST	29 IU/L
ALT	24 IU/L
LDH	220 IU/L
γ-GTP	72 IU/L
ALP	385 IU/L
CHE	112 IU/L
T-cho	164 mg/dL
TP	6.1 g/dL
Alb	3.4 g/dL
BUN	15 mg/dL
Cre	0.62 mg/dL
Na	141 mEq/L
K	4.2 mEq/L
Cl	108 mEq/L
Ca	8.6 mEq/L

Hb: Hemoglobin; Hct: Hematocrit; Plt: Platelet; PT: Prothrombin time; HPT: Hepaplastin test; AT3: Antithrombin III; CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; ICG-R15: Indocyanine green retention rate at 15 min; CRP: C-reactive protein; T-Bil: Total bilirubin; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; LDH: Lactate dehydrogenase; γ-GTP: Gamma-glutamyl transpeptidase; ALP: Alanine phosphatase; CHE: Cholinesterase; T-cho: Total cholesterol; TP: Total protein; Alb: Albumin; BUN: Blood urea nitrogen; Cre: Creatinine.

benefit[30,31]. However, in the NORDIC7 trial, patients received FLOX plus cetuximab, and in the COIN trial, patients received FOLFOX or XELOX (capecitabine + L-OHP) plus cetuximab. On the other hand, in the CELIM study[32], the response rates were significantly higher in patients whose tumors were wild type for KRAS (46 of 67 patients, 70%) than in those with KRAS mutations (11 of 27 patients, 41%). The R0 resection rates were 38% *vs* 30% (25 of 52 *vs* 18 of 44 patients) for FOLFOX plus cetuximab *vs* FOLFIRI plus cetuximab, respectively. Therefore, we selected FOLFOX plus cetuximab as the chemotherapy regimen.

It is quite difficult to predict the response to the tumor with chemotherapy. Negri *et al*[33] and Catalano *et al*[34] reported that mucinous histology predicts for poor response rate and overall survival in patients with colorectal cancer with fluorouracil-based or oxaliplatin-based chemotherapy. In our case, the histology of the original site was well-to-moderately differentiated adenocarcinoma. It might lead a good response with mFOLFOX6 plus cetuximab.

To secure the future remnant liver volume, PTPE may be considered as an option in selected patients[35-37].

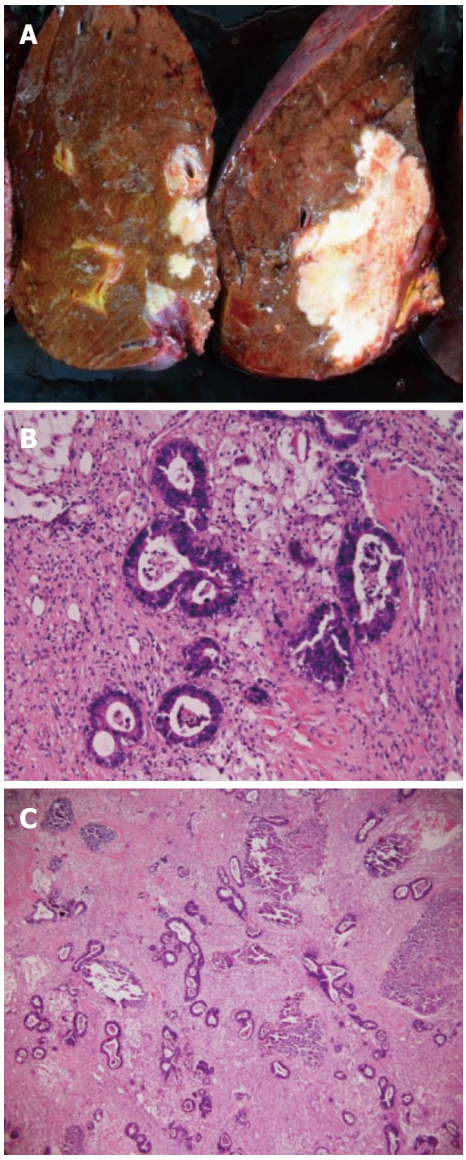

Figure 3 Resected specimen. A: Cut surface. The tumor was 70 mm × 40 mm in size. The tumor was grayish-white and stony hard; B, C: Hematoxylin and eosin (HE) staining of the resected specimen; B: HE staining, × 400. Adenocarcinoma; C: HE, × 100. Approximately 50% of the adenocarcinoma was necrotic.

The purpose of preoperative PTPE is to initiate compensatory hypertrophy in the future remnant liver in an attempt to counteract liver failure after major hepatectomy[38-40]. Nagino *et al*[41] underlined that indocyanine green clearance of the future liver remnant after PTPE should be more than 0.05 for major hepatectomy in patients with biliary cancers. However, liver function of our case was damaged due to 6 courses of FOLFOX. As an indication of PTPE for the patient with chemotherapy associated steatohepatitis remains controversial, it is to be elucidated.

There is no evidence on how many courses are the most effective for facilitating surgical resection in patients responsive to chemotherapy. It has been reported that the median duration of response to

FOLFOX ranges from 4 to 6 courses[21,42]. Use of more than 6 courses of L-OHP-based chemotherapy is significantly associated with SOS[24]. Among patients undergoing a major hepatectomy, SOS was associated with significantly higher morbidity and longer hospital stays[24]. Therefore, preoperative chemotherapy was performed for total of 6 courses.

In conclusion, we herein report a case successfully treated with a multidisciplinary strategy, consisting of conversion chemotherapy using FOLFOX plus cetuximab and PTPE. This strategy may contribute to improve resectability for initially unresectable CRLM, thus leading to prolonged survival.

COMMENTS

Case characteristics

A 72-year-old female with a high level of carcinoembryonic antigen (CEA) was referred to our hospital.

Clinical diagnosis

The patient had no clinical symptoms.

Differential diagnosis

A high level of CEA are associated with adenocarcinoma; colon cancer, stomach cancer, lung cancer, pancreatic cancer, and so on.

Laboratory diagnosis

Laboratory workup showed that CEA level increased to 27.0 ng/mL.

Imaging diagnosis

Computed tomography revealed a synchronous colorectal liver metastasis, which involved the right hepatic vein and the inferior vena cava.

Pathological diagnosis

Histopathological analysis resulted in the diagnosis of a well-to-moderately differentiated adenocarcinoma, and the cancer cells were found to have wild-type KRAS.

Treatment

The patient underwent laparoscopic sigmoidectomy, followed by 5-fluorouracil, leucovorin, and oxaliplatin plus cetuximab, portal vein embolization for future remnant liver volume, and a right hemihepatectomy.

Term explanation

Conversion chemotherapy is a method that liver resection becomes possible by intensive chemotherapy, in patients with initially unresectable colorectal liver metastases (CRLM).

Experiences and lessons

In selected patients with unresectable metastases, CRLM may be down-staged by systemic chemotherapy with or without molecular targeted therapy, so that liver resection may be completed.

Peer-review

Conversion chemotherapy might lead to a successfully curative liver resection in a patient with initially unresectable synchronous CRLM. However, it depends on patients if systemic chemotherapy is effective.

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CASE REPORT

Rapidly aggravated skeletal muscle metastases from an intrahepatic cholangiocarcinoma

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Author contributions: Lee J and Lee SW designed the research; Lee J and Kim SY collected the patient's clinical data; Han SY and Baek YH analyzed the data; Lee J and Rhyou HI wrote the paper.

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Abstract

We present a rare case of intrahepatic cholangiocarcinoma (ICC) with multiple skeletal muscle metastases. The patient was a 55-year-old Asian woman presenting with abdominal pain; abdominal and pelvic computed tomography and magnetic resonance cholangiopancreatography revealed an unresectable ICC with hepatic metastasis and metastastatic lymphadenopathy in the porto-caval area. After 3 mo of treatment with palliative radiotherapy and chemotherapy, magnetic resonance imaging of the thoracolumbar spine detected right psoas muscle and paraspinous muscle metastases. We performed an ultrasound-guided percutaneous fine-needle biopsy that confirmed a similar pattern of poorly differentiated adenocarcinoma. The patient treated with palliative chemotherapy and achieved 10 mo of survival. Here we report the first case quickly spread to multiple sites of muscle even though the three-month treatment, compare to the other cases reported muscle metastases at diagnosis.

Key words: Intrahepatic cholangiocarcinoma; Skeletal muscle metastasis; Adenocarcinoma

Core tip: The case presented with multiple skeletal muscle metastases from an intrahepatic cholangiocarcinoma which only 5 cases have been reported so far.

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INTRODUCTION

An intrahepatic cholangiocarcinoma (ICC) is a malignant tumor that originates in the small bile ducts. The risk factors for cholangiocarcinoma are primary sclerosing cholangitis, biliary-duct cysts, hepatolithiasis, liver fluke infections, and conditions such as hepatitis B infection, hepatitis C infection, obesity, and cirrhosis[1]. Surgical resection is the only treatment

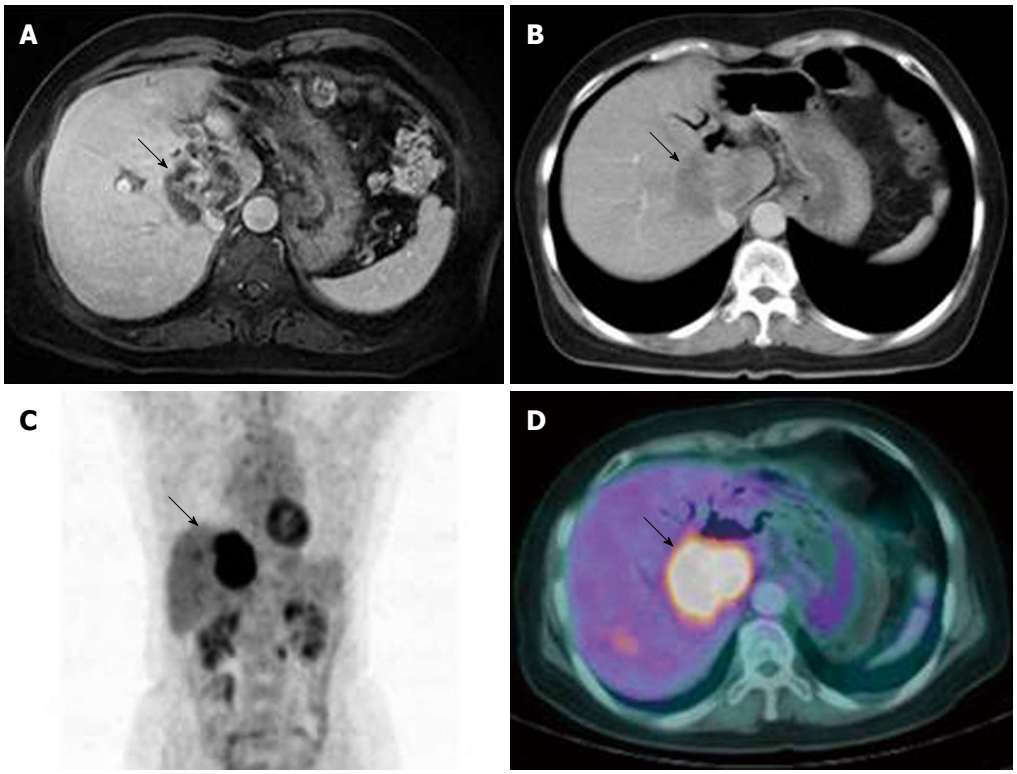

Figure 1 Computed tomography, magnetic resonance cholangiopancreatography, and positron-emission tomography-computed tomography showing an intrahepatic cholangiocalcinoma in the liver at first diagnosis. A: Computed tomography axial scan shows an ill-defined, low-attenuation mass in segments S1 and S5 of the liver; B: Contrast-enhanced, T1-weighted, axial image shows the 6-cm, peripherally enhanced mass in segments S1, S5, and S8; C and D: In the positron-emission tomography-computed tomography images, an area of strong hypermetabolism is noted (arrows), corresponding to the 6-cm soft tissue mass in the liver (maximum standardized uptake value, 10.4).

modality providing a chance for cure; the reported median survival, after surgical resection, ranges from 16 to 46 mo, with a 5-year survival rate of 21%-25%[2]. If the tumor is unresectable, the median survival is less than 1 year. In this setting, patients may need palliative therapies such as surgical or endoscopic biliary drainage, chemotherapy, radiation therapy, radiofrequency ablation, hepatic intraarterial chemotherapy, photodynamic therapy or stenting[3].

Common metastatic sites for ICC are the regional lymph nodes (hilar, peripancreatic, and periaortic lymph nodes) and adjacent organs. Hematogenous ICC metastases commonly occur to distant organs, such as the lungs, bones, and adrenal glands, but skeletal muscle metastasis is unusual for any malignancy. Here, we describe an unusual case of distant skeletal muscle metastasis from an ICC.

CASE REPORT

A 55-year-old Asian woman presented to our gastroenterology department with abdominal pain. Ultrasound and contrast-enhanced computed tomography (CT) examinations of her abdomen and pelvis, reported by the referring hospital, showed an ill defined, low-density lesion in segments S1, S5, and S8 of liver (Figure 1A). The patient's past medical history included cholecystectomy with Roux-en-Y hepaticojejunostomy because of an intrahepatic duct stone, and common hepatic duct stones in 1995. In 2009, she had undergone coil-embolization for an unruptured aneurysm(12 mm × 9 mm × 10 mm) of her left internal carotid artery communicating segment. She denied tobacco or alcohol use, and her family medical history was unremarkable.

Upon admission, a physical examination determined her blood pressure (100/60 mmHg), pulse (70/min), respiration rate (20/min), and temperature (36.6 ℃). Upon examination, the patient was not icteric but had right upper quadrant abdominal tenderness. Laboratory tests revealed her white blood cell 6330/μL (3000-9300/μL), platelet 214000/μL (140000-360000/μL), hemoglobin 11.9 g/dL (11-15 g/dL), blood urea nitrogen 24 mg/dL (8-26 mg/dL), creatinine 0.8 mg/dL (0.7-1.0 mg/dL), aspartate aminotransferase 32 IU/L (0-35 IU/L), alanine aminotransferase 29 IU/L (10-35 IU/L), alkaline phosphatase 589 IU/L (104-338 IU/L), total bilirubin 0.6 mg/dL (0.2-1.2 mg/dL), alpha-fetoprotein 4.54 ng/mL (0-15 ng/mL), carcinoembryonic antigen 9.45 ng/mL (0-8 ng/mL), and carbohydrate antigen 19-9 3.01 U/mL(0-37 U/mL). All hepatic viral markers were negative, as was her chest radiograph. Magnetic resonance cholangiopancreatography (MRCP) showed a 5.7 cm, heterogeneous hepatic mass in S1, S5, and S8, and multiple hepatic metastases in the right lobe (Figure

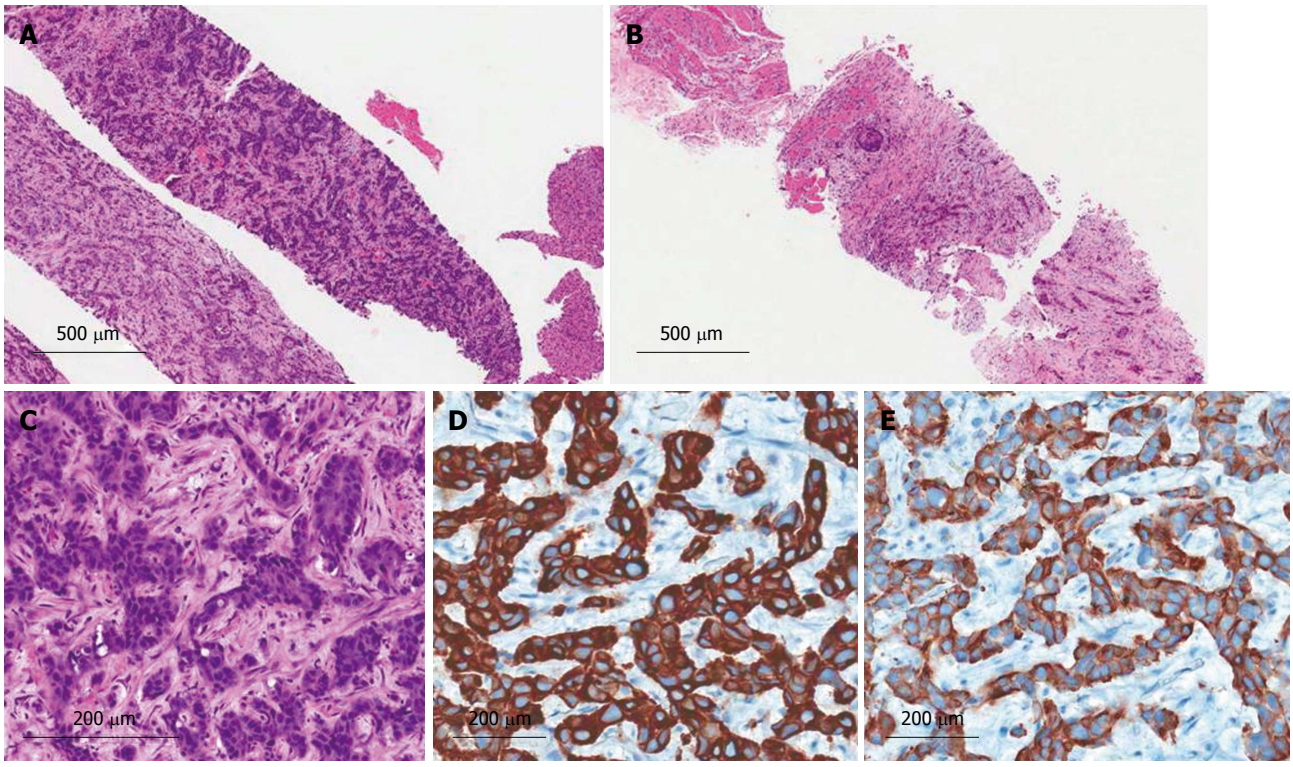

Figure 2 Histopatholgic findings. A and C: Liver shows a poorly differentiated cholangiocarcinoma. (hematoxylin and eosin, x 40, x 400); B: Skeletal muscle tissue shows metastasis of a poorly differentiated cholangiocarcinoma (hematoxylin and eosin, x 40); D and E: Neoplastic glands were positive in cytokeratin 7 and cytokeratin 19 staining (immunostain, x 400).

1B). An ultrasound-guided percutaneous needle biopsy of the low-echoic liver mass was performed; histopathology confirmed a poorly differentiated adenocarcinoma (Figure 2A and C), which was positive for cytokeratins 7 and 19 (Figure 2D and E). Positron-emission tomography-computed tomography (PET-CT) detected a 6 cm, hypermetabolic, intrahepatic malignancy involving S1, adjacent to the right lobe of the liver (maximum standardized uptake value, 10.41), multiple hepatic metastases in the right lobe, and metastatic lymphadenopathy in the porto-caval, common hepatic, and left para-aortic areas (Figure 1C and D).

The patient received palliative three-dimensional conformal radiotherapy, with a total dose of 4750 cGy in 19, 250 cGy fractions and concurrent chemotherapy with monthly intra-arterial floxuridine (0.3 mg/kg per day, days 1-14). After 3 mo of treatment, she complained of low back pain; magnetic resonance imaging (MRI) of her thoracolumbar spine revealed metastatic masses in both the paraspinous and right psoas muscles (Figure 3A and B). An ultrasound-guided percutaneous needle biopsy of the low-echoic, paraspinous muscle confirmed a metastatic poorly differentiated adenocarcinoma (Figure 2B). PET-CT showed multiple, distant, metastatic hypermetabolic masses in her paravertebral area, left upper arm, bilateral periscapular area, anterior and posterior chest walls, bilateral psoas muscles, lower anterior abdominal wall, bilateral gluteal muscles, and bilateral proximal thigh muscles (Figure 3C-F). The patient was then subjected to chemotherapy with once every 3 wk gemcitabine (1000 mg/m^2 per day) and cisplatin (100 mg/m^2 per day) but died 10 mo after diagnosis of this progressive disease.

DISCUSSION

Distant metastases to skeletal muscle are rare, despite the skeletal muscle accounting for approximately 50% of the total body mass and its abundant vascularization[4]. The reason for this low metastatic rate is not well understood. The effects of lactic acid on tumor cell production[5], and the influence of variable and turbulent blood flow, β-adrenergic stimulation, tissue oxygen levels, and host immune responses may provide protection to the skeletal muscle[6]. Moreover, protease inhibitors in the extracellular matrix of the muscle may also help prevent cancer cell invasion[7].

However, recent improvements in radiological procedures may facilitate a more frequent diagnosis of skeletal muscle metastasis. CT, MRI, ultrasound, 67 Ga scintigraphy, and PET-CT are useful for establishing a diagnosis of intramuscular metastasis. CT provides an accurate means of evaluating and defining the extent of skeletal muscle tumors and their relationship to adjacent bones, fascial planes, vessels, and fat. MRI is the gold standard for imaging of skeletal muscle disease and is a valuable modality in the detection of skeletal muscle metastasis[8]. T1-

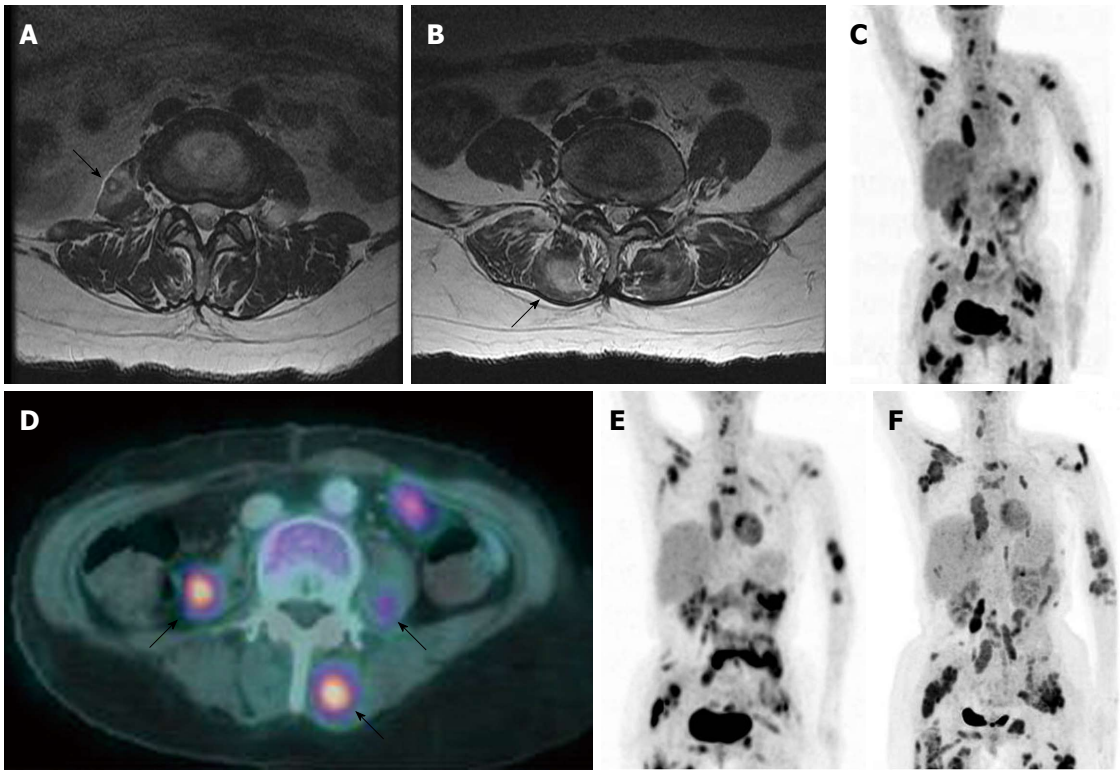

Figure 3 Thoracolumbar spine magnetic resonance imaging and positron-emission tomography-computed tomography images show multiple skeletal metastases in a whole body scan after 3 mo of palliative radiochemotherapy from first diagnosis. A and B: T2-weighted, axial images show ill-defined, high signal intensity masses in the psoas and paraspinal muscles; C and D: Positron-emission tomography-computed tomography (PET-CT) images show multiple masses with hypermetabolism in the paravertebral area, left upper arm, bilateral periscapular area, anterior and posterior chest walls, bilateral psoas muscles, lower anterior abdominal wall, bilateral gluteal muscles, and both proximal thigh muscles; E and F: Follow-up PET-CT images show aggravated multiple muscle metastasis after 3 and 7 mo of gemcitabine-cisplatin combination chemotherapy.

Table 1 Clinical features of patients with skeletal muscle metastasis

Patient No. (Ref.)	Age (yr)/sex	Ethnicities	Primary origin CC	Metastatic sites	Treatment	Follow-up (mo)	Outcome
1[14]	48/F	NA	Extrahepatic-	Rectus femoris	Resection	8	Dead
2[15]	45/M	Asian	Intrahepatic	Paraspinal muscle in thorax, buttock	Chemotherapy, Radiation	4	Dead
3[4]	44/M	Asian	Intrahepatic-	Bilateral psoas and spinal erector muscles	NA	NA	NA
4[16]	69/W	NA	Extrahepatic-	Psoas and iliacus muscles	Radiation	2	Dead
5[17]	72/M	NA	NA	Calf muscle	Resection	NA	NA

CC: Cholangiocarcinoma; NA: Not applicable.

weighted images of skeletal muscle metastasis have a low- to iso-intense signal, and there is high signal intensity in T2-weighted images[9,10]. Since these findings are not specific for soft tissue metastasis of carcinomas, pathological examinations of biopsy specimens provide the most useful findings for differentiating soft tissue metastasis of a carcinoma from soft tissue sarcomas and abscesses.

Treatment guidelines have not been established for patients with carcinomas and distant skeletal muscle metastases. External beam radiation, chemotherapy, surgical resection of localized disease, and combination therapies have been considered for their potential improvement of patient symptoms, without any significant prolongation of survival. Hence, the prognosis for carcinoma patients with distant metastases to the skeletal muscle is poor, with a median survival of less than 12 mo[11-13].

To our knowledge, only 5 other cases of metastases to skeletal muscle from cholangiocarcinoma have been reported in the world literature (Table 1).

In conclusion, we describe the first patient rapidly metastasized multiple sites of muscle from ICC even though the three-month palliative radiochemotherapy, compare to the other cases reported muscle metastases at diagnosis.

COMMENTS

Case characteristics

A 55-year-old Asian woman with a history of cholecystectomy with roux-en-Y hepaticojejunostomy because of IHD stones presented with abdominal pain.

Clinical diagnosis

On physical examination, the patient had tenderness over right upper quadrant of abdomen.

Differential diagnosis

Hepatocellularcarcinoma, cholangiocarcinoma, metastatic carcinoma

Laboratory diagnosis

WBC 6330/uL, AFP 4.54 ng/mL, CEA 9.45 ng/mL, and CA 19-9 3.01 U/mL; liver function test were within normal limits.

Imaging diagnosis

Computed tomography (CT)/positron-emission tomography (PET) scan showed 5.7 cm, heterogeneous hepatic mass in S1, S5, and S8 with SUV of 10.4, and follow-up PET-CT showed multiple, distant, metastatic hypermetabolic masses in her paravertebral area, left upper arm, bilateral periscapular area, anterior and posterior chest walls, bilateral psoas muscles, lower anterior abdominal wall, bilateral gluteal muscles, and bilateral proximal thigh muscles.

Pathological diagnosis

Ultrasound guided liver biopsy and muscle biopsy revealed a poorly differentiated adenocarcinoma of cytokeratins 7 and 19 positive.

Treatment

The patient was treated with concurrent chemotherapy(intra-arterial floxuridine) and radiation therapy (4750 cGy in 19, 250 cGy fractions) and then palliative Gemcitabine-cisplatin after progression.

Related reports

Only 5 other cases of metastatic carcinoma to muscle have been reported and the pathogenesis and treatment has not been established.

Term explanation

An intrahepatic cholangiocarcinoma (ICC) is a malignant tumor that originates in the small bile ducts and rarely metastasized to skeletal muscle.

Experiences and lessons

This case report represents rapidly progressed to multiple muscle metastasis from ICC, compare to the other cases reported muscle metastases at diagnosis.

Peer-review

The case report of a "rapidly aggravated skeletal muscle metastases from an intrahepatic cholangiocarcinoma" is very exciting data to the reader and may provide the novel important information of this cancer. However, the manuscript writing didn't focus and summarize the results to support the conclusion. Moreover, many points should be concerned before consideration to publish.

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CASE REPORT

Retreatment with peginterferon and ribavirin in chronic hepatitis C

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Abstract

The development of boceprevir and telaprevir was a major step forward in the treatment of chronic hepatitis C. In addition, the treatment of these infections has been recently revolutionized by the approval of sofosbuvir and simeprevir. However, there are several challenges associated with the application of novel drugs, such as new and more frequent adverse events, new drug interactions, and excessively high treatment costs. An additional concern is viral resistance. These considerations highlight the fact that direct-acting antiviral agents are not a panacea and may not be the best option for all patients who are in need of therapy. This retrospective study revealed that the sustained virologic response was not significantly reduced following peginterferon and ribavirin retreatment compared with the new therapy. We suggest that patients who experience relapse shortly after completing treatment with peginterferon and ribavirin have a reasonable chance of achieving a sustained virologic response when retreated with these drugs alone.

Key words: Chronic hepatitis C; Direct-acting antiviral agents; Peginterferon; Ribavirin; Retreatment

Core tip: Chronic hepatitis C-infected patients who experience relapse shortly after completing treatment with peginterferon and ribavirin have a reasonable chance of achieving a sustained virologic response when retreated with these drugs alone. Thus, it would be very reasonable to proceed with peginterferon and ribavirin retreatment alone, particularly in patients with factors associated with high rates of sustained virologic response, such as a low viral load at relapse (< 400000 IU/mL) and an early virologic response at week 12 of retreatment.

Jo YM, Lee SW, Han SY, Baek YH, Kim SY, Kim WJ, Ahn JH, Lee JY. Retreatment with peginterferon and ribavirin in chronic hepatitis C. *World J Gastroenterol* 2015; 21(6): 1994-1999 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i6/1994.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i6.1994

INTRODUCTION

Hepatitis C is an infection caused by a viral attack on the liver, and leads to inflammation and chronic liver disease. The long-term consequences of hepatitis C virus (HCV) infection are minimal changes, chronic hepatitis, cirrhosis, and hepatocellular carcinoma[1-3]. From 1995-2000, the overall prevalence of HCV infection among Koreans over 40 years of age was estimated to be 1.29%[4]. Approximately 25% of chronically infected patients ultimately progress to cirrhosis and other complications[5-8].

The aim of HCV treatment is to achieve sustained eradication of the virus and prevent progression to cirrhosis and related complications[9]. Sustained virologic response (SVR) is the term used for successful treatment of HCV, and refers to the viremia 24 wk after completion of antiviral therapy[10,11].

Initial treatment for chronic hepatitis C is carried out using a combination of conventional interferon-alpha and ribavirin over a period of 24-48 wk according to the patient's genotype. Interferon-alpha has progressively been replaced by peginterferon, which has emerged as the most effective regimen[12].

The recommended treatment for chronic hepatitis C infection consisted of combination therapy with peginterferon and ribavirin until May 2011, when the US Food and Drug Administration (FDA) licensed the first direct-acting antiviral agents that directly impeded viral replication. In clinical trials of chronic hepatitis C patients receiving peginterferon and ribavirin combined with boceprevir or telaprevir, SVR has been accomplished in 63%-75% of treatment-naïve patients, in 69%-88% of peginterferon and ribavirin relapsers, and in up to 33% of peginterferon and ribavirin non-responders[13-16]. Recently, the FDA has approved sofosbuvir and simeprevir for the treatment of chronic hepatitis C as components of a combination treatment regimen.

Triple therapy is connected with increased adverse events, and thus requires closer patient observation compared with the previous treatment. Additionally, boceprevir and telaprevir may induce HCV-resistant mutations, and it is likely that cross-resistance to direct-acting antiviral agents will emerge in some patients who are without SVR[17,18]. The clinical impacts of resistance to sofosbuvir and simeprevir have not been well-established.

The aim of this retrospective study was to assess the efficacy of peginterferon and ribavirin therapy in patients with chronic hepatitis who have relapsed following an initial course of peginterferon-based therapy to facilitate the development of a novel treatment for HCV infection.

CASE REPORT

Case 1

The patient in this case was a 37-year-old male with a history of intravenous drug abuse. HCV infection was diagnosed in September 2008 on the basis of amplification of HCV RNA genotype 1b. Serum HCV RNA level was 585026 IU/mL at baseline. It was suggested that his HCV infection was caused by intravenous drug abuse. Physical examination was unremarkable. The serum aspartate aminotransferase (AST) level was 56 IU/L. Serum alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (GGT), and alkaline phosphatase (ALP) were normal. The bilirubin, serum creatinine, and prothrombin time (PT) were normal. Liver function was reported as Child-Turcotte-Pugh (CTP) class A. Liver ultrasonography indicated chronic liver disease with mild splenomegaly.

In January 2009, combination therapy with peginterferon alpha-2a and ribavirin was initiated with the informed consent of the patient. Peginterferon alpha-2a was administered subcutaneously at a weekly dose of 180 μg together with 1200 mg/d ribavirin for 48 wk. Serum HCV RNA levels were determined at baseline and at week 12 by quantitative PCR. The patient accomplished complete early virologic response (EVR). Additionally, he accomplished an end-of-treatment response (ETR) at week 48.

However, in March 2010, at 15 wk after the completion of treatment, the reappearance of serum HCV RNA was documented. The serum HCV RNA level was 13367 IU/mL. Immediately after virologic relapse was documented, combination therapy with peginterferon alpha-2b and ribavirin was initiated as retreatment. A weekly dose of 120 μg peginterferon alpha-2b was administered subcutaneously together with 1200 mg/d ribavirin for 12 wk. The patient did not achieve a rapid virologic response (RVR) at week 4 of retreatment, but he did achieve complete EVR at week 12. Additionally, undetectable serum HCV RNA was determined at week 24 by qualitative PCR. The patient attained SVR at 24 wk following the discontinuation of retreatment.

Case 2

The patient in this case was a 58-year-old male with a chronic hepatitis C-infected spouse. HCV infection was diagnosed in February 2007 on the basis of amplification of HCV RNA genotype 1b. The serum HCV RNA level was 3420000 IU/mL at baseline, as determined by quantitative PCR. It was suggested that his HCV infection had been transmitted by sexual intercourse. Physical examination was unremarkable. The serum ALT level was 54 IU/L and the serum AST level was normal. The GGT level was 103 IU/L. The ALP level was normal. The serum creatinine and total bilirubin were normal. Liver function was reported as CTP class A. Liver ultrasonography indicated chronic liver disease with mild splenomegaly.

In February 2007, combination therapy with peginterferon alpha-2b and ribavirin was initiated with the informed consent of the patient. A weekly dose

of 100 μg of peginterferon alpha-2b was administered subcutaneously together with 1200 mg/d of ribavirin for 29 wk. This patient tolerated the ribavirin well. Because of leukopenia, peginterferon alpha-2b was administered at a weekly dose of 80 μg for the remaining treatment period. Serum HCV RNA levels were determined at baseline and at weeks 4 and 12 by quantitative PCR. Because of his partial EVR, undetectable serum HCV RNA was confirmed at week 24. The patient achieved ETR at week 48 of therapy.

However, in May 2008, at 11 wk after the end of antiviral therapy, the reappearance of serum HCV RNA was documented. The serum HCV RNA level was 536 IU/mL, as determined by quantitative PCR assay. Immediate virologic relapse was documented, and combination therapy with peginterferon alpha-2b and ribavirin was initiated as retreatment. Peginterferon alpha-2b was administered at a weekly dose of 80 μg together with a 1200 mg/d ribavirin for 12 wk. The patient did not accomplish RVR at week 4 of retreatment, but did achieve complete EVR at week 12. Additionally, undetectable serum HCV RNA was determined at week 24. The patient achieved SVR at 24 wk following the discontinuation of retreatment.

Case 3

The third patient was a 60-year-old male with a chronic hepatitis C-infected spouse. HCV infection was diagnosed in April 2007 on the basis of amplification of HCV RNA genotype 1b. The serum HCV RNA level was 7710000 IU/mL at baseline, as determined by quantitative PCR. It was suggested that his HCV infection was transmitted by sexual intercourse. Physical examination was unremarkable. The serum AST level was 78 IU/L. The ALT, GGT, and ALP were normal. The PT, bilirubin, and serum creatinine were normal. Liver function was reported as CTP class A. Liver ultrasonography indicated chronic liver disease with mild splenomegaly.

In April 2007, combination therapy with peginterferon alpha-2b and ribavirin was initiated with the informed consent of the patient. Peginterferon alpha-2b was administered at a weekly dose of 80 μg together with a daily dose of 1000 mg of ribavirin for 48 wk. Serum HCV RNA levels were determined at baseline and at weeks 4 and 12 by quantitative PCR. The patient did not achieve RVR, but did achieve a partial EVR. Because he did not attain complete EVR, undetectable serum HCV RNA was determined at week 24 by qualitative PCR. The patient attained an ETR at week 48 of therapy.

However, in May 2008, at 12 wk after the completion of antiviral therapy, the reappearance of HCV RNA in his serum was determined by qualitative PCR and a serum ALT level of 45 IU/L. Virologic relapse was immediately documented and peginterferon alpha-2b and ribavirin combination therapy was initiated as retreatment. Peginterferon alpha-2b was administered subcutaneously at a weekly dose of 80 μg together with 1000 mg/d ribavirin for 12 wk. The serum ALT levels were 16, 23, and 20 IU/L at weeks 4, 12, and 24 of retreatment, respectively. This patient did not accomplish RVR at week 4 of retreatment, but did accomplish complete EVR at week 12. Additionally, undetectable serum HCV RNA was determined at week 24 by qualitative PCR. This patient attained SVR at 24 wk following the discontinuation of retreatment.

Case 4

The fourth patient was a 40-year-old male with a chronic hepatitis C-infected spouse. HCV infection was diagnosed in July 2006 on the basis of amplification of HCV genotype 2a/2c. The serum HCV RNA level was 113000 IU/mL at baseline. It was suggested that his HCV infection was transmitted by sexual intercourse. Physical examination was unremarkable. The serum AST was 56 IU/L. The ALT, GGT, and ALP were normal. The PT, bilirubin, and serum creatinine were normal. Liver function was reported as CTP class A. Liver ultrasonography indicated chronic liver disease with mild splenomegaly.

In October 2006, combination therapy with peginterferon alpha-2b and ribavirin was initiated with the informed consent of the patient. Peginterferon alpha-2b was administered at a weekly dose of 120 μg together with 800 mg/d of ribavirin for 24 wk. Serum HCV RNA levels were determined at baseline and at weeks 4 and 12 by quantitative PCR. This patient did not achieve RVR, but did achieve complete EVR. However, he lacked an ETR at week 24 of therapy. The serum HCV RNA level was 155 IU/mL, as determined by quantitative PCR.

Combination therapy with peginterferon alpha-2a and ribavirin was immediately initiated as retreatment. Peginterferon alpha-2a was subcutaneously administered at a weekly dose of 180 μg together with 800 mg/d of ribavirin for 12 wk. The patient did not accomplish RVR at week 4 of retreatment, but did accomplish complete EVR at week 12. Additionally, undetectable serum HCV RNA was determined at week 24 by qualitative PCR. He accomplished SVR at 24 wk following interruption of retreatment.

The baseline characteristics and antiviral therapy regimens of these patients are presented in Table 1.

DISCUSSION

The first-line treatment for chronic hepatitis C was peginterferon-ribavirin treatment until May 2011, when the first direct-acting antiviral agents were licensed by the FDA for use with peginterferon and ribavirin in treatment-naïve and treatment-experienced HCV-infected patients with compensated liver cirrhosis. HCV-infected patients following the addition of the first direct-acting antiviral agents to combined peginterferon and ribavirin treatment accomplished

Table 1 Baseline characteristics of patients

Characteristics	Case 1	Case 2	Case 3	Case 4
Gender	Male	Male	Male	Male
Age (yr)	37	58	60	40
Body weight (kg)	80	69	63	84
Height (m)	1.75	1.69	1.65	1.84
Body mass index (kg/m^2)	26.1	24.2	23.1	24.8
HCV genotype	1b	1b	1b	2a/2c
HCV infection route	Intravenous drug abuse	Sexual intercourse	Sexual intercourse	Sexual intercourse
Serum HCV RNA level at baseline (IU/mL)	585026	3420000	7710000	113000
Antiviral therapy regimen	Peginterferon α-2a and ribavirin for 48 wk ↓ Peginterferon α-2b and ribavirin for 12 wk	Peginterferon α-2b and ribavirin for 48 wk ↓ Peginterferon α-2b and ribavirin for 12 wk	Peginterferon α-2b and ribavirin for 48 wk ↓ Peginterferon α-2b and ribavirin for 12 wk	Peginterferon α-2b and ribavirin for 48 wk ↓ Peginterferon α-2a and ribavirin for 12 wk

HCV: Hepatitis C virus.

higher SVR rates compared with peginterferon-ribavirin treatment. Recently, the FDA approved sofosbuvir and simeprevir for the treatment of chronic hepatitis C. The addition of direct-acting antiviral agents to combined peginterferon and ribavirin treatment represents a significant advancement in HCV treatment[13-16].

Non-responders to peginterferon-based therapy or those who relapse following this therapy are increasing in number, with such individuals displaying decompensated liver cirrhosis. Before the availability of direct-acting antiviral agents, limited retreatment options were available for these patients. Recently, retreatment with peginterferon and ribavirin plus a direct-acting antiviral agent has been shown to lead to a higher SVR rate compared with peginterferon-ribavirin treatment[15-18].

The development of direct-acting antiviral agents marks a major step towards the eventual aim of more potent and shorter courses of treatment, and other compounds are also being developed with different viral targets. This is a rapidly-changing era in HCV treatment, with such major developments being achieved as new compounds that can cooperate with clinicians to manage this hard-to-cure virus.

A new era of treatment for HCV is dawning with the development of direct-acting antiviral agents, but these new agents are not a magic bullet. Unfortunately, clinical trials have recognized that the use of these new agents in isolation leads to the prompt emergence of viral resistance and mutations[19,20].

The onset of the acquired immune deficiency syndrome pandemic led to antiviral drugs with diverse mechanisms being developed. However, human immunodeficiency virus (HIV) remains unconquered due to its viral resistance. Many properties of HCV are similar to that of HIV. Thus, resistance can be the primary scourge of anti-HCV treatment.

Almost all patients will experience treatment related adverse events that lead to poor tolerability, which can in turn result in early treatment interruption. The addition of direct-acting antiviral agents to peginterferon-based treatment is connected by adverse events, and thus requires the interruption of direct-acting antiviral agents in 10%-12% of patients[21]. Adverse events that occur with increased frequency in subjects receiving direct-acting antiviral agents include anemia, leukopenia, taste disorder, gastrointestinal discomfort, fatigue, skin eruption, and perianal discomfort[13-16].

These considerations highlight the fact that direct-acting antiviral agents are not a cure-all and may not be the best choice for all patients who need treatment.

This retrospective study revealed that retreatment with peginterferon-ribavirin treatment may be of value in some patients in whom previous peginterferon and ribavirin combination therapy has failed.

After the completion of initial antiviral treatment, patients are monitored to assess their treatment response and the occurrence of adverse events. Laboratory monitoring includes measurements of white blood cell count, aminotransferase, serum creatinine, and HCV RNA at 4, 8, 12, and 24 wk after end of treatment. Patients with virologic relapse are immediately retreated with peginterferon and ribavirin. This retrospective study indicated that the SVR was not significantly decreased in patients retreated with peginterferon and ribavirin compared with the new therapy.

Pre-retreatment predictors of response may be helpful for informing patients of their probability of SVR. SVR rates were higher in treatment-naïve patients with a viral load of less than 400000 IU/mL[11]. Likewise, the results of this study clearly demonstrate that viral load at relapse is very important in predicting the outcome of retreatment. The changes in the HCV RNA levels in these patients are presented in Table 2.

The absence of EVR is the most powerful means

Table 2 Changes in hepatitis C virus RNA level (IU/mL)

Weeks of treatment	Previous treatment					Retreatment	
	0	12	24	48	0	12	36
Case 1	585026	< 50	Negative	Negative	13367	< 50 (complete EVR)	Negative (SVR)
Case 2	3420000	63	Negative	Negative	536	< 50 (complete EVR)	Negative (SVR)
Case 3	7710000	226	Negative	Negative	Positive[1]	< 50 (complete EVR)	Negative (SVR)
Case 4	113000	< 50	Positive	N/A	155	< 50 (complete EVR)	Negative (SVR)

[1]Positive of hepatitis C virus RNA was determined by qualitative PCR. EVR: Early virologic response; SVR: Sustained virologic response.

of identifying non-responders in treatment-naïve patients[22,23]. All patients achieving complete EVR also achieved SVR in this study. The outcomes of this study clearly show that complete EVR is very important in predicting the outcome of retreatment (Table 2).

There is no common consent regarding the retreatment period for chronic hepatitis C-infected patients who have previously relapsed. Almost all patients treated with peginterferon-ribavirin treatment have experienced adverse events. Adverse events represent a major cause for patients giving up on the treatment. Therefore, the optimal duration of retreatment should be based on virologic clearance to promote the adherence of patients to the regimen. In this study, after peginterferon plus ribavirin was administered for 12 wk, patients achieved complete EVR at week 12 of retreatment and SVR at 24 wk following discontinuation of retreatment.

The evolution of compounds that inhibit virus replication by inhibiting either HCV protease or polymerase will refine the treatment of hepatitis C. Many such drugs are currently under development. New drugs promise to increase the SVR rates for chronic hepatitis C-infected patients and possibly shorten treatment duration. However, this enhanced response comes with an increased incidence of adverse events and a higher cost. An additional concern with regard to newer therapies is that of viral resistance. The emergence of resistant variants has not been observed with the current peginterferon and ribavirin therapy. In addition, adherence to the new therapeutic regimens cannot be omnipotent.

Retreatment with peginterferon and ribavirin plus a direct-acting antiviral agent in chronic hepatitis C-infected patients has led to higher SVR rates compared with those achieved with previous treatment in clinical trials. SVR was achieved in 69%-88% of relapsers and in 29%-33% of null responders[13-16]. This retrospective study determined that SVR was not significantly reduced by the peginterferon and ribavirin combination therapy compared with the new therapy.

Collectively, we suggest that patients who relapse shortly after completing treatment with peginterferon plus ribavirin have a reasonable chance of achieving SVR when retreated with peginterferon and ribavirin alone. It would be very reasonable to proceed with this retreatment, particularly in those patients possessing factors connected by high rates of SVR, such as a low viral load at relapse (< 400000 IU/mL) and complete EVR at week 12 of retreatment

New direct-acting antiviral agents cannot be the best retreatment option for motivated patients who have previously relapsed. When making the decision to treat using a new therapy, the clinician must consider the benefits of the simpler, less-toxic regimen connected by lower SVR rate with the new therapy and its associated higher toxicity, complexity, increased risk of resistance development, and potentially higher SVR rate.

A limitation of our study is that there were insufficient numbers of patients to strongly substantiate our findings. Secondly, information regarding liver histology and interleukin 28B gene polymorphism was not reported.

COMMENTS

Case characteristics

Case 1: A 37-year-old male with a history of chronic hepatitis C virus (HCV) infection caused by intravenous drug abuse. Case 2: A 58-year-old male with a chronic hepatitis C-infected spouse; it was suggested that his HCV infection had been transmitted by sexual intercourse.

Clinical diagnosis

HCV was diagnosed on the basis of amplification of HCV RNA.

Differential diagnosis

Viral hepatitis, drug-induced hepatitis, autoimmune hepatitis, and steatohepatitis.

Laboratory diagnosis

Case 1: HCV RNA level of 585026 IU/mL. Case 2: HCV RNA level of 3420000 IU/mL.

Imaging diagnosis

Case 1: Liver ultrasonography showed early liver cirrhosis with splenomegaly. Case 2: Liver ultrasonography showed chronic liver disease with mild splenomegaly.

Treatment

The two patients were treated with peginterferon and ribavirin.

Related reports

There is no consensus on a retreatment method for patients with HCV who have previously relapsed.

Term explanation

Sustained virologic response is the absence of HCV RNA in the blood at 24 wk after treatment completion.

Experiences and lessons

These findings suggest that patients who relapse shortly after completing treatment with peginterferon plus ribavirin have a reasonable chance of SVR when retreated with the previous treatment.

Peer-review

This article discusses chronic hepatitis C retreatment methods in patients who have previously relapsed.

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CASE REPORT

Successful treatment of complex cholangiolithiasis following orthotopic liver transplantation with interventional radiology

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Author contributions: Zhou CG and Zhai RY designed the study; Wei BJ and Gao K were the patient's attending doctors; Dai DK, Wei BJ and Gao K performed interventional procedures; Zhou CG and Wei BJ organized the report; Zhou CG wrote the paper.

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Abstract

Bile duct stones are a serious and the third most common complication of the biliary system that can occur following liver transplantation. The incidence rate of bile duct stones after liver transplantation is 1.8%-18%. The management of biliary stones is usually performed with endoscopic techniques; however, the technique may prove to be challenging in the treatment of the intrahepatic bile duct stones. We herein report a case of a 40-year-old man with rare, complex bile duct stones that were successfully eliminated with percutaneous interventional techniques. The complex bile duct stones were defined as a large number of bile stones filling the intra- and extrahepatic bile tracts, resulting in a cast formation within the biliary tree. Common complications such as hemobilia and acute pancreatitis were not present during the perioperative period. The follow-up period was 20 mo long. During the postoperative period, the patient maintained normal temperature, and normal total bilirubin and direct bilirubin levels. The patient is now living a high quality life. This case report highlights the safety and efficacy of the percutaneous interventional approach in the removal of complex bile duct stones following liver transplantation.

Key words: Complex bile duct stones; Percutaneous interventional technique; Liver transplantation; Intrahepatic bile tract; Extrahepatic bile tract

Core tip: Bile duct stones are a rare, but serious complication that can occur following liver transplantation and can be very difficult to treat with endoscopic techniques. A case is presented involving a 40-year-old man who developed complex bile duct stones following liver transplantation, with a large number of stones filling the intra- and extrahepatic bile tracts and forming a cast in the biliary tree. The patient's stones were successfully removed using percutaneous interventional techniques, highlighting the safety and efficacy of this approach in the removal of complex bile duct stones following liver transplantation.

Zhou CG, Wei BJ, Gao K, Dai DK, Zhai RY. Successful treatment of complex cholangiolithiasis following orthotopic

liver transplantation with interventional radiology. *World J Gastroenterol* 2015; 21(6): 2000-2004 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i6/2000.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i6.2000

INTRODUCTION

Liver transplantation is the only effective method for the treatment of terminal-stage chronic liver diseases. However, there is a high incidence of biliary complications following liver transplantation and long-term, repeated therapies, as the biliary epithelium appears to be more susceptible to ischemia than the liver parenchyma[1,2]. Biliary stones, though uncommon, are a serious complication of orthotopic liver transplantation, which, if not properly treated, may result in severe infection of the biliary tract, liver failure, and even graft loss. The incidence of biliary stones in liver transplant patients varies widely from 1.8%-18%[3-5]. Biliary stones are sometimes managed with interventional radiology, surgery or retransplantation, but most commonly treated with endoscopy[6-9]. The disadvantage of endoscopy is a potential for the development of serious complications such as hemobilia and acute pancreatitis. Furthermore, endoscopy is technically more challenging in the treatment of intrahepatic bile stones. Following stone extraction there is a recurrence rate of 17% within a median of six months[10,11].

Percutaneous interventional techniques represent an alternative approach for the treatment of bile duct stones. These techniques provide a safe and effective approach for the treatment of not only extrahepatic, but also intrahepatic, bile stones. Here, we present a case of rare, complex bile duct stones following orthotopic liver transplantation. The bile duct stones were considered complex, as a large number were found within the intra- and extrahepatic bile tracts, forming a cast in the biliary tree. The removal of bile duct stones was performed successfully using percutaneous interventional techniques. This case report provides an alternative option for managing difficult and serious complications following liver transplantation.

CASE REPORT

In May 2009, a 40-year-old man underwent classic orthotopic liver transplantation for the treatment of end-stage liver disease secondary to hepatocellular carcinoma from liver cirrhosis. The patient's post-transplantation recovery was uneventful; however, three months after the surgery his skin and sclera gradually became yellow. The primary diagnosis was obstructive jaundice, which was confirmed by magnetic resonance cholangiopancreatography (MRCP) and cholangiography, and treated with percutaneous transhepatic biliary drainage. In August 2011, due to persistent aggravated jaundice, the patient was referred to our section for further treatment.

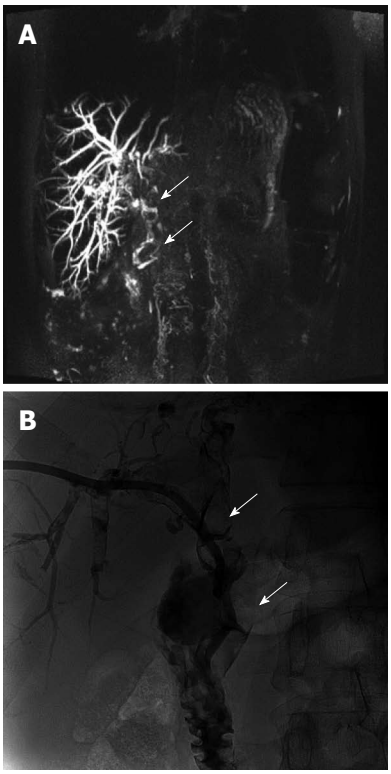

Figure 1 Imaging of the intra- and extrahepatic bile ducts. A: Magnetic resonance cholangiopancreatography showing a large number of bile duct stones within the intra- and extrahepatic bile ducts (arrows) with a mild dilation of the intrahepatic bile duct; B: Percutaneous transhepatic cholangiography showing the bile duct cast, which is a result of molding of the bile ducts by the stones (arrows).

The patient's medical history showed presence of both hepatitis B and liver cirrhosis. The laboratory examination revealed: total bilirubin, 140 μmol/L (normal, 3.4-20.5 μmol/L); direct bilirubin, 125 μmol/L (normal, 0-6.8 μmol/L); alkaline phosphatase, 152 U/L (normal, 50-136 U/L); gamma-glutamyl transpeptidase, 77 U/L (normal, 5-85 U/L); hepatitis B surface antigen, negative; alpha-fetoprotein, 2.5 ng/mL (normal, 0-13.6 ng/L); and normal total cholesterol and triglyceride levels. The MRCP showed presence of a large number of bile duct stones in the intra- and extrahepatic bile ducts with a mild dilation of the intrahepatic bile duct. Percutaneous transhepatic cholangiography (PTC) showed molding of the intra- and extrahepatic bile ducts into a duct cast by the stones (Figure 1).

Interventional procedures

Informed consent was obtained from the patient prior to the interventional procedures. The first procedure was carried out on September 20, 2011. First, a hydrophilic, coated guidewire was used to traverse the stenosis section of the biliary anastomotic stricture entering into the jejunum. Second, an 8 mm

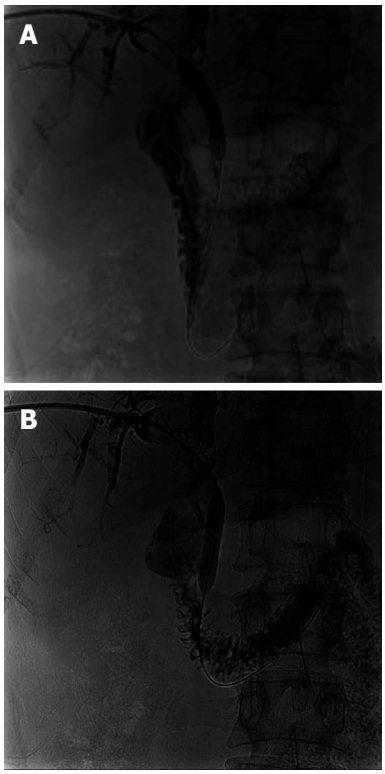

Figure 2 Crushing of the extrahepatic bile duct stones. Percutaneous transhepatic cholangiography showing that bile duct stones were crushed from the A: Proximal; B: Distal duct with the inflated balloon catheter.

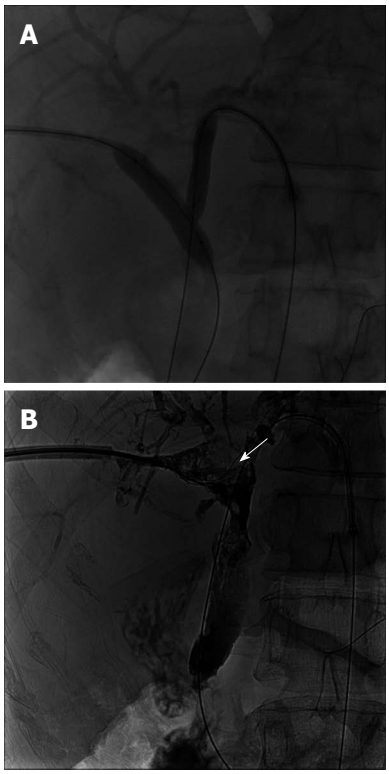

Figure 3 Percutaneous interventional techniques used on the intrahepatic bile stones. A: The intrahepatic bile stones were crushed with the double balloon catheter technique; B: The large residual stones were cut with the basket extractor (arrow).

× 40 mm balloon catheter (ATB5-35-40-8-4.0; COOK Inc., Bloomington, IN, United States) was placed over the guidewire at the extrahepatic bile duct. Consequently, the extrahepatic bile duct stones were crushed from the proximal to distal bile duct using the inflated balloon catheter (Figure 2). Finally, a biliary tunnel was formed in the biliary cast, and the stone fragments were flushed with a 12 F biliary drainage tube. The procedure was carried out two more times afterwards. The inflated balloon catheter was used to push some of the bigger fragments to the duodenum *via* balloon sphincteroplasty. On December 5, 2011, a double balloon catheter technique was used for efficient elimination of the intrahepatic bile stones and left side percutaneous transhepatic biliary drainage was performed. Balloon catheters [8 mm × 40 mm and 10 mm × 40 mm (ATB5-35-40-10-4.0; COOK Inc.)] were placed in the left- and right-side bile ducts and simultaneously inflated in order to crush the intrahepatic bile stones. On December 28, 2011, a basket extractor (Nitinol Tipless Stone Extractor, NTSE-030115-UDH-MB; COOK Inc.) was used to cut some of the large residual stones (Figure 3).

Four months after the percutaneous transhepatic biliary drainage, PTC showed no presence of bile duct stones in either the intra- or extrahepatic or bile ducts. The intrahepatic bile duct was not dilated while the extrahepatic bile duct was mildly dilated (Figure 4). Hence, the left-side drainage tube was removed on April 16, 2012 while the right-side drainage tube was removed on May 23, 2012. All of the procedures were free from complications. The follow-up period was 20 mo (until February 2014). During the post-operative period, the patient maintained normal body temperature, as well as normal levels of total and direct bilirubin. The patient is now able to live a normal life.

DISCUSSION

Orthotopic liver transplantation is currently the standard therapy for end-stage liver diseases. Despite great improvements in organ preservation, immunosuppression, and surgical techniques, biliary complications (BCs), including non-anastomotic and anastomotic strictures, stone formation, and bile leaks, remain a common source of morbidity. Consequently, patients may undergo long-term and repeated therapies including percutaneous, endoscopic, and surgical procedures. BCs may also increase liver retransplantation rates and influence graft and patient survival rates. The overall incidence of BCs after orthotopic liver transplantation is between 5% and 25%[6]. Although the underlying reasons for the bile stone formation are unknown, some research indicates that anastomotic strictures, bacterial infections, ischemia, elevated total cholesterol, and

Figure 4 Elimination of bile duct stones. Percutaneous transhepatic cholangiography showed no presence of the bile stones and a normal biliary tree four months after the percutaneous interventional procedures.

triglyceride levels could predispose a patient to the formation of biliary stones or sludge[5,6,12].

This case report presents a rare occurrence of complex bile stones following orthotopic liver transplantation and their successful elimination using the percutaneous interventional technique. The case presented a serious situation in which both the intra- and extrahepatic bile ducts were filled with a large number of stones, making their removal by endoscopy difficult. Hence, a series of percutaneous interventional techniques were used in the treatment of the patient. The extrahepatic bile duct stones were crushed with the inflated balloon catheter, and the stone fragments were flushed by a drainage tube. Some of the large stone fragments were pushed into the duodenum *via* balloon sphincteroplasty. The intrahepatic bile stones were eliminated with the double-balloon catheter technique and a basket extractor was used to cut the large residual stones. The biliary drainage tube was reserved, and ursodeoxycholic acid was administrated to promote biliary excretion and avoid biliary tract infection. Four months later, the bile stones were completely eliminated, and normal liver function was recovered with no perioperative complications. The patient had no clinical symptoms, and the laboratory examinations were normal at the last check-up. The patient is now living a high quality of life.

In conclusion, the percutaneous interventional technique provides a safe and effective treatment of bile stones following orthotopic liver transplantation without commonly associated complications, such as hemobilia or acute pancreatitis. The case presented here demonstrates that the percutaneous interventional technique is especially suitable for the removal of complex bile stones, which are otherwise difficult to treat endoscopically.

COMMENTS

Case characteristics

A 40-year-old male patient with a history of orthotopic liver transplantation presented with aggravated jaundice.

Clinical diagnosis

A large number of bile duct stones filled both the intra- and extrahepatic bile ducts with mild dilation of the intrahepatic bile duct.

Differential diagnosis

Anastomotic stenosis, common bile duct stone, and malignant obstructive jaundice.

Laboratory diagnosis

Total bilirubin, 140 μmol/L; direct bilirubin, 125 μmol/L; alpha-fetoprotein, 2.5 ng/mL.

Imaging diagnosis

Magnetic resonance cholangiopancreatography showed a large number of bile duct stones in the intra- and extrahepatic bile ducts with an associated mild dilation of the intrahepatic bile duct.

Treatment

The patient was treated with a series of percutaneous interventional procedures.

Related reports

An endoscopy is commonly used in the management of biliary stones; however, endoscopy may be technically challenging in the treatment of the intrahepatic bile stones.

Term explanation

Complex bile duct stones are defined as a large number of bile stones within the intra- and extrahepatic bile tracts forming a cast in the biliary tree.

Experiences and lessons

This case report emphasizes the safety and efficacy of the percutaneous interventional approach in the removal of complex bile duct stones following liver transplantation.

Peer-review

This article applies a percutaneous interventional technique in the elimination of complex bile duct stones following orthotopic liver transplantation.

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