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

World Journal of *Gastroenterology*

World J Gastroenterol 2014 August 28; 20(32): 11023-11466

Published by Baishideng Publishing Group Inc

World Journal of Gastroenterology

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

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2014-2017

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INDEXING/ABSTRACTING

World Journal of Gastroenterology is now indexed in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifier, and Directory of Open Access Journals. ISI, Journal Citation Reports®, Gastroenterology and Hepatology, 2013 Impact Factor: 2.433 (36/74); Total Cites: 20957 (6/74); Current Articles: 1205 (1/74); and Eigenfactor® Score: 0.05116 (6/74).

FLYLEAF I-IX Editorial Board

NAME OF JOURNAL
World Journal of Gastroenterology

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

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

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PUBLISHER
Baishideng Publishing Group Inc
8226 Regency Drive,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

PUBLICATION DATE
August 28, 2014

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

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DOI: 10.3748/wjg.v20.i32.11023

World J Gastroenterol 2014 August 28; 20(32): 11023-11032
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

EDITORIAL

Non-pulmonary allergic diseases and inflammatory bowel disease: A qualitative review

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Supported by NIH Intramural Program to Kotlyar DS; NCI Fellowship Program, NIH
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Received: September 29, 2013 Revised: February 16, 2014
Accepted: April 14, 2014
Published online: August 28, 2014

Abstract

While the etiological underpinnings of inflammatory bowel disease (IBD) are highly complex, it has been noted that both clinical and pathophysiological similarities exist between IBD and both asthma and non-pulmonary allergic phenomena. In this review, several key points on common biomarkers, pathophysiology, clinical manifestations and nutritional and probiotic interventions for both IBD and non-pulmonary allergic diseases are discussed. Histamine and mast cell activity show common behaviors in both IBD and in certain allergic disorders. IgE also represents a key immunoglobulin involved in both IBD and in certain allergic pathologies, though these links require further study. Probiotics remain a critically important intervention for both IBD subtypes as well as multiple allergic phenomena. Linked clinical phenomena, especially sinonasal disease and IBD, are discussed. In addition, nutritional interventions remain an underutilized and promising therapy for modification of both allergic disorders and IBD. Recommending new mothers breastfeed their infants, and increasing the duration of breastfeeding may also help prevent both IBD and allergic diseases, but requires more investigation. While much remains to be discovered, it is clear that non-pulmonary allergic phenomena are connected to IBD in a myriad number of ways and that the discovery of common immunological pathways may usher in an era of vastly improved treatments for patients.

Key words: Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Food intolerance; Food allergies; Biomarkers; Pathophysiology; Nutrition; Probiotics

Core tip: There are multiple clinical, pathophysiological and therapeutic commonalities between nonpulmonary allergic disease and inflammatory bowel disease (IBD). In particular, in terms of pathophysiology, histamine expression is upregulated in both IBD and allergic diseases. Ulcerative colitis, in particular, shows upregulation of the Th2 pathway which is seen in a large number of allergic phenomena including sinonasal disease. Both probiotics and nutritional interventions are promising therapies for both IBD and allergic disease (especially food intolerance, food allergies, and eczema) but these require more investigation. Recommending mothers breastfeed their infants, and for a longer duration also shows potential promise in prevention of both IBD and food allergies, but also requires further study.

Kotlyar DS, Shum M, Hsieh J, Blonski W, Greenwald DA. Non-pulmonary allergic diseases and inflammatory bowel disease: A qualitative review. *World J Gastroenterol* 2014; 20(32): 11023-11032 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11023.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11023

INTRODUCTION

Inflammatory bowel disease (IBD) is comprised of two major disorders, ulcerative colitis and Crohn's disease (CD). The exact pathophysiology of IBD remains unclear; however immune dysregulation plays a substantial role, with likely significant involvement of the Th1 and Th17 pathways in Crohn's and the Th2 pathway in ulcerative colitis. Intriguingly, some clinical manifestations of allergic disorders and IBD overlap, as well as expression of selected cytokines and immune responses. In particular, both disorders feature histamine release and IgE overexpression. Certain probiotics have been found to be useful in both disorders. There have been some studies that have shown a correlation between sinonasal allergic disease and IBD. Moreover, some food allergies and intolerances have been linked to IBD. Finally, sulfasalazines are often used to treat patients with IBD; such therapy can require desensitization for an individual patient to successfully use. It is evident that allergic disorders and IBD share common etiological characteristics and also share potential common treatment pathways.

HISTAMINE AND OTHER BIOMARKERS ASSOCIATED WITH IBD AND ALLERGY

Role of histamine and mast cells

In 1978, Dvorak *et al*[1], followed by Levo and Livni[2], found ultrasonographic and morphological evidence of degranulation of mast-cells in the submucosa of ileal specimens from patients with CD. They found release of mediators including histamine, platelet activation factor, and eosinophil chemotactic factor, all of which may play a role in the pathophysiology of CD. Subsequently, Dvorak *et al*[1], using transmission electron microscopic studies discovered a markedly increased number of mast cells that were located in the edematous submucosa and between smooth muscle cells in the ileum of subjects with CD. In addition, evidence of degranulation of mast-cells, basophils and eosinophils in the affected area of ileum was also observed[1]. Similarly, an increased number of mast-cells with intense degranulation was found in the active stage of ulcerative colitis (UC)[3]. Moreover, it was also demonstrated that the median number of mast-cells in normal colonic tissue was significantly greater in patients with UC than controls (patients examined for colonic adenomas) or those with CD (1500 per milligram of tissue *vs* 1250 per milligram of tissue, $P < 0.05$)[4]. Furthermore, median mast-cell counts obtained from inflamed colonic tissue were significantly ($P < 0.01$) greater than the number of mast-cells in non-inflamed tissue in patients with IBD (2000 per milligram of tissue *vs* 1500 per milligram of tissue in UC and 1700 per milligram of tissue *vs* 1250 per milligram of tissue in CD)[4]. On the other hand, King *et al*[5] showed an increased mean number of mast cells (19.5) at the demarcation line between active and inactive areas of colonic inflammation in 12 of 20 (60%) UC patients. Finally, a Japanese group determined that patients with IBD or collagenous colitis had a greater number of mast cells in the upper part of the lamina propria of the colon than healthy controls and that patients with IBD had a higher number of mast cells in the lower part of the lamina propria of the colon as compared to those with collagenous colitis and healthy controls[6].

Knuston *et al*[7] observed that patients with CD of the distal ileum had a significantly greater mean histamine secretion rate within the small intestine than did healthy controls (152 ng/cm *vs* 71 ng/cm small intestine per hour, $P < 0.01$), and that histamine secretion was related to disease activity (active disease defined as CDAI > 150: 193 ng/cm per hour *vs* inactive disease defined as CDAI < 150: 105 ng/cm per hour). Further study also suggested that histamine secretion was significantly increased in inflamed colonic mucosa in patients with both CD and UC when compared to their non-inflamed colonic mucosa or colonic mucosa in healthy controls[8]. A more recent study showed that urinary excretion of *N*-methylhistamine was significantly increased in patients with active IBD when compared to inactive IBD or non-IBD controls and such urinary histamine excretion strongly correlated with endoscopic activity of CD measured by the CD Endoscopic Index of Severity ($r^2 = 0.70$, $P < 0.0001$)[9]. Greater expression of tumor necrosis factor-α (TNF-α) by mast cells was also found in the submucosa and muscularis propria of the ileum in patients with CD when compared to controls; significantly greater numbers of TNF-α-labeled mast cells were noted in the muscularis propria both in uninflamed (1.7-fold, $P < 0.05$) and in inflamed ileum (4.6-fold, $P < 0.002$)[10]. In addition, TNF-α expression was found to be greater in the submucosa in inflamed *vs* uninflamed ileum in CD patients (1.6-fold, $P < 0.01$), while it was lower in the lamina propria in inflamed *vs* uninflamed ileum in CD patients (0.4-fold, $P < 0.05$)[10]. This is noteworthy as TNF-α has been shown to be an important factor in the inflammatory cascade leading to the inflammatory response in the murine model for IBD[11].

IgE

IgE as a biomarker of disease activity in IBD: It has been suggested that IgE may mediate an allergic response in patients with IBD. Evidence supporting this hypothesis includes the presence of peripheral and tissue eosinophilia in IBD patients[12,13], increased numbers of mast cells or cells containing IgE in rectal mucosa of patients with IBD[14,15], concomitant atopic disease in patients with IBD[16,17] and a good response to disodium cromoglycate in IBD patients[18-20].

Several studies have assessed IgE levels in patients with IBD. Pepys *et al*[21] suggested that some patients with IBD (25% of UC patients and 31% of CD patients) may

have elevated serum IgE levels. These data were further supported by Levo *et al*[22], who claimed that patients with IBD have significantly increased mean serum level of IgE when compared to healthy controls (358 IU/mL *vs* 103 IU/mL, $P < 0.05$). On the other hand, several studies report normal serum IgE levels in IBD patients[23-26]. Becker *et al*[25] determined that specific serum IgE levels to food allergens such as egg white, whole milk, β-lactoglobulin and wheat were undetectable in IBD patients and thus suggested that the allergic hypothesis of IBD should be rejected. However, a prior study by Mee *et al*[26] observed a significantly higher frequency of positive reactions to food allergens using the skin prick test in IBD patients when compared to healthy controls.

Role of IgE in desensitization to therapies for IBD: Desensitization to sulfasalazine (SASP) has been found to be effective in patients who experienced hypersensitivity reactions to SASP[27-30]. This is achieved by administration of successively larger doses of SASP, thereby allowing the presence of specific IgE in a controlled fashion without causing massive histamine release from mast cells[29]. In the largest published study, reporting on 47 patients with IBD, desensitization was successful in 85% of patients with IBD, and there was no recurrence of hypersensitivity reactions in 82.5% of those who were successfully desensitized[29]. In addition, among the successfully desensitized patients, 100% of UC patients and 78% of CD patients remained in long-term remission on SASP alone or in combination with intermittent prednisone[29]. Caution is advised in attempting desensitization in patients with agranulocytosis, toxic epidermal necrolysis, or fibrosing alveolitis[30]. The risks of these severe reactions may outweigh the benefit of continuing to take SASP containing medications.

PROBIOTICS

At a workshop held at Yale University in 2007, recommendations were made with regards to the use of probiotics in clinical settings for a variety of indications, including IBD[31]. For IBD, the recommendations included a class "A" recommendation (defined as one made on "strong, positive, well-conducted controlled" studies) for the use of VSL#3 for the prevention and maintenance of remission in pouchitis[31]. In addition, some probiotics were given a class "C" recommendation (one "based on some positive studies") for IBD[31]. These included VSL#3 for the induction of remission of pouchitis, and for inducing remission and maintenance of remission in ulcerative colitis[31]. The probiotic *Lactobacillus* GG (LGG) was noted to be beneficial in both IBD and allergic diseases; LGG has a "C" class recommendation in the treatment of CD, and a class "A" designation treatment of atopic eczema[31]. Probiotics may also help with cow milk allergy[31]. See Tables 1-3 for other disease indications and probiotic regimens, with additional recommendations from the workshop in 2007.

Lactobacillus also has specific bacteriocidal effects, including against *Lactococcus*, *Streptococcus*, *Staphylococcus*, *Listeria* and *Mycobacteria*[32]. The bacterium uses two different pathways to neutralize competing bacteria. First, it uses a small molecule to inhibit growth of competitor bacteria, likely a short chain fatty acid, and also uses hydrogen peroxide to alter the bacterial microenvironment from an aerobic one to an anerobic one[32]. In addition, Lactobacillius also has immunomodulatory abilities. First, the bacillus increases transepithelial resistance, and it also upregulates the key toll-like receptors TLR2 and TLR9. These toll-like receptors recognize foreign hostile viral and bacterial antigens and their activation causes a large expansion of cytokine expression and amplifies the immune response against hostile antigens[32].

Table 1 Recommendations for probiotic use

Clinical condition	Effectiveness	Organism
Diarrhea		
Infectious-adult-treatment	A	Saccharomyces boulardi, LGG
Infectious-childhood-treatment	A	LGG. *Lactobacillus reuteri*
Prevention of infection	B	*S. boulardii*, LGG
Prevention of AAD	A	*S. boulardii*, LGG, *L. casei*, *L. bulgaricus*, *S. thermophilus*
Treatment of recurrent CDAD	B	*S. boulardii*, LGG
Prevention of CDAD	B	LGG, *S. boulardii*
IBD		
Pouchitis		
Preventing and maintaining remission	A	VSL#3
Induce remission	C	VSL#3
Ulcerative colitis		
Inducing remission	C	*Escherichia coli* Nissle, VSL#3
Maintenance	C	*E. coli* Nissle, VSL#3
Crohn's	C	*E. coli* Nissle, *S. boulardii*, LGG
IBS	B	*Bifidobacterium infantis*
IBS	C	*Bifidobacterium animalis*, VSL#3, *Lactobacillus plantarum*
Immune response	A	LGG, *Lactobacillus acidophilus*, *L. plantarum*, *Bifidobacterium lactis*, *Lactobacillus johnsonii*
Allergy		
Atopic eczema assoc. with cow milk allergy		
Treatment	A	LGG, *B. lactis*
Prevention	A	LGG, *B. lactis*
Radiation enteritis	C	VSL#3, *L. acidophilus*
Vaginosis and vaginitis	C	*L. acidophilus*, LGG, *L. reuteri*

Reproduced with permission from reference Floch *et al*[31]. An "A" recommendation is based on strong, positive, well-conducted, controlled studies in the primary literature, not abstract form; A "B" recommendation is based on positive, controlled studies but the presence of some negative studies; A "C" recommendation is based on some positive studies. IBD: Inflammatory bowel disease; LGG: *Lactobacillus* GG; *S. boulardii*: *Saccharomyces boulardii*.

ATOPY, NASAL DISEASE AND URTICARIA IN IBD

Prior studies have described a relationship between IBD

Table 2 Results of random controlled trials which reported efficacy of probiotics in patients with inflammatory bowel disease

Probiotic	Situation	Control	No. of subjects	Duration (mo)	Relapse probiotic *vs* control	Ref.
E. coli Nissle 1917	Ulcerative colitis	5-ASA	120	4	16% *vs* 11.3%	[70]
E. coli Nissle 1917	Ulcerative colitis	5-ASA	120	12	67% *vs* 73%	[71]
E. coli Nissle 1917	Crohn's disease	Placebo	28	12	30% *vs* 70%[a]	[72]
VSL#3	Pouchitis	Placebo	40	9	15% *vs* 100%[a]	[73]
VSL#3	Prevention of pouchitis	Placebo	40	12	10% *vs* 40%[a]	[74]
VSL#3	Crohn's disease[1]	5-ASA	28	12	20% *vs* 40%[a]	[75]
S. boulardii	Ulcerative colitis	5-ASA	31	12	30% *vs* 35%[a]	[76]
S. boulardii	Crohn's disease	5-ASA	28	6	6.3% *vs* 37.5%[a]	[77]

Reproduced with permission from reference Marteau[69]. [1]Postoperative. [a]$P < 0.05$ *vs* control. 5-ASA: 5-aminosalicylic acid; *E. coli*: *Escherichia coli*; *S. boulardii*: *Saccharomyces boulardii*.

Table 3 Probiotics in treatment of inflammatory bowel disease: Randomized controlled trials

Entity	Ref.	Population (*n*)	Probiotic(s)	Treatment group	Effect
Crohn's disease	Bousvaros *et al*[79]	Children (75; age, 5-21 yr)	LGG	Maintenance of remission	No benefit
	Plein *et al*[80]	Adults (20)	*S. boulardii*	Maintenance of remission	Reduced diarrhea *vs* placebo
	Malchow[72]	Adults (28)	*E. coli* Nissle 1917	Maintenance of remission	No benefit
	Prantera *et al*[81]	Adults (45)	LGG	Maintenance of surgically induced remission	No benefit
	Schultz *et al*[82]	Adults (11)	LGG	Treatment of active Crohn's disease	No benefit
Ulcerative colitis	Kruis *et al*[70]	Adults (120)	*E. coli* Nissle 1917	Maintenance of medically induced remission	Equal to mesalamine
	Rembacken *et al*[71]	Adults (120)	*E. coli* Nissle 1917	Maintenance of medically induced remission	Equal to mesalamine
	Kruis *et al*[83]	Adults (327)	*E. coli* Nissle 1917	Maintenance of medically induced remission	Equal to mesalamine
	Ishikawa *et al*[84]	Adults (21)	*B. breve* and *B. bifidum* and *L. acidophilus* YIT 0168	Maintenance of medically induced remission	Superior to placebo
Pouchitis	Gionchetti *et al*[73]	Adults (40)	VSL#3	Maintenance of antibiotic-induced remission of chronic pouchitis	Superior to placebo
	Mimura *et al*[85]	Adults (36)	VSL#3	Maintenance of antibiotic-induced remission of chronic pouchitis	Superior to placebo
	Gionchetti *et al*[86]	Adults (40)	VSL#3	Maintenance of antibiotic-induced remission of chronic pouchitis	Superior to placebo
	Kuisma *et al*[87]	Adults (20)	LGG	Treatment of active pouchitis	No benefit

Reproduced with permission from reference Szajewska *et al*[78]. LGG: *Lactobacillus* GG; *E. coli*: *Escherichia coli*; *S. boulardii*: *Saccharomyces boulardii*; *B. breve*: *Bifidobacterium Breve*; *B. bifidum*: *Bifidobacterium bifidum*; *L. acidophilus*: *Lactobacillus acidophilus*.

and allergy. It is unclear whether this association is manifested as atopy (consisting of atopic dermatitis, allergic rhinitis, asthma, and food allergy) or nasal disease. The association was hypothesized because patients with allergy have an abnormal IgE antibody response to common environmental antigens and earlier findings of peripheral and tissue eosinophilia in patients with IBD had suggested an IgE mediated response. Furthermore, because some allergic symptoms were associated with other systemic inflammatory disorders, it was felt that patients with colitis might also have an increased likelihood of developing atopic illnesses.

A review of prior studies has shown that these results may be equivocal and may only be pertinent in relation to food allergies and possibly sinonasal disease. One study showed that in children with atopic eczema, food allergy is associated with increased intestinal inflammation, as manifested by elevated levels of fecal eosinophil cationic protein, TNF-α and α1 antitrypsin[33]. Similarly, in another study, while there was no correlation observed between frequency of personal history of atopy, serum IgE levels and prick test response between IBD patients and controls, it was observed that IBD patients had a higher frequency of positive prick test when tested against food allergens[26]. There also seemed to be a positive relationship between IBD and chronic sinonasal disease, since the prevalence of chronic sinonasal disease was elevated in patients with IBD, specifically in patients with CD and especially in those also with obstructive bowel complications[34]. Interestingly, nearly 70% of these patients had some degree of sinonasal disease; see Figure 1 for a graph depicting the prevalence of sinonasal disease in patients with IBD.

Other atopic features (asthma, hay fever and allergic rhinitis) were investigated in patients with UC and CD with the results indicating that atopic features were twice as common in patients with UC, but no different between patients with CD and controls, suggesting that hypersensitivity may play a part in UC but not in CD[17].

In contrast, other studies found no association be-

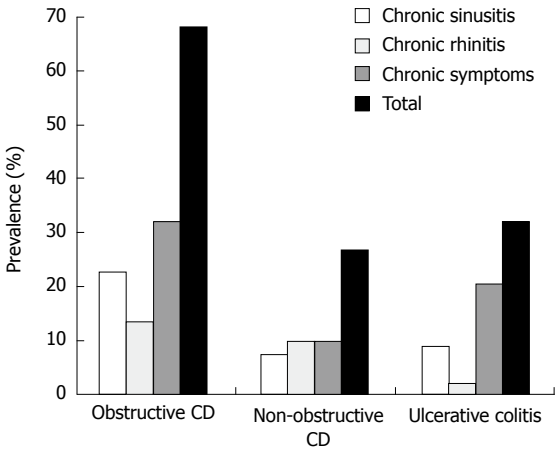

Figure 1 Prevalence of sinonasal disease in inflammatory bowel disease patients. Reproduced with permission from reference Book *et al*[34]. CD: Crohn's disease.

tween controls and IBD patients in terms of atopic symptoms. Personal history analyses for atopic symptoms (asthma, allergic rhinitis, eczema, urticaria, and allergic symptoms) were assessed in another study where the prevalence of atopy with skin-prick tests (using five allergens - mixed grass pollens, dog hair, cat fur, *Dermatophagoides pteronyssinus* and *Aspergillus fumigatus*) and serum IgE concentrations among 122 patients with IBD and 103 age-matched controls was examined[35]. The authors showed no statistical difference in the percentage of patients with positive skin tests between controls and IBD patients and also no difference between the subgroups of IBD patients (CD, ulcerative colitis, and ulcerative proctitis)[35].

Another study assessed skin test reactivity in IBD patients. In this study, two different populations were examined, one in the United States and another in Czechoslovakia, and patients were classified as having CD or UC by clinical, pathological and radiological criteria; those who had been treated with immunosuppressives were taken off these medications for three weeks. The authors found no evidence of skin test anergy (assessed by using multiple antigens - candida, mumps, PPD, streptokinase-streptodornase, and trichophytin) when all patients were compared to controls[36].

Despite initial studies that suggested an association between IBD and IgE mediated allergic reactions, direct evidence is still lacking and the role of IgE mediated reactions remains unclear.

NUTRITION AND IBD

Epidemiology of food intolerance and IBD

IBD is characterized by chronic inflammation of the gastrointestinal tract. The etiology of IBD is complex and probably multi-factorial. Nutrition is an important modulator of IBD[37]. In particular, it is felt that relationships between food antigens and immune pathways may alter the course of IBD[38]. Gut bacteria and the inflammatory response are altered by the ingestion of differing foods[39].

Most patients with IBD are intolerant to selected (or specific) food items. Food intolerance has been evaluated in multiple studies. In a survey administered to 132 patients (along with 70 controls) with IBD, food intolerance was reported by 66% of patients with CD, and 64% of UC patients[40]. The most common symptoms included diarrhea and abdominal pain[40]. In a study that evaluated the antibody response to cow's milk antigen, it was found that IgG and IgM antibodies to beta-lactoglobulin were significantly elevated in patients with UC and CD when compared with normal subjects[41]. Elevation of IgG further correlated with involvement of the ileum, increase in inflammatory markers, and was higher in untreated patients; interestingly there was no change in IgM levels after sulfasalazine or steroid therapy[41]. Another study reported on a questionnaire examining dietary habits, the amount of food consumed, and whether patients had problems with specific foods[42]. A total of 122 foods were evaluated. Intolerance to chocolate, dairy products, fats and artificial sweeteners were seen in both UC and CD, and patients with CD seemed to have a greater range of problems with specific foods[42]. From 80%-100% of bacteria in the colonic flora of CD patients are bound by immunoglobulin whereas, in controls only 20% are bound; when enteral feeds are given the percentage bound in CD significantly decreases[43,44].

The prevalence of food reactions was studied in 375 adult patients attending a gastroenterology outpatient clinic: 32% complained of food allergies as being the source of their abdominal complaints and in 14.4% laboratory testing was consistent with intestinal food allergy[45]. Laboratory testing included counts of eosinophils, the presence of specific IgE against food antigens, increased total IgE, specific clinical signs of atopy, and cromoglycate sensitivity[45]. Confirmation of the diagnosis of food allergy was found in 3.2%[45]. This was confirmed through elimination diet and subsequent rechallenge or allergen provocation testing during EGD[45].

Breastfeeding may have a protective effect against developing IBD[37,46]. Of thirteen reported case-control studies, 3/13 (23.1%) found that patients with IBD were less likely to have been breast fed as compared to controls[37]. In another study of 308 matched patients, the patients with CD were found to have had an average breast-feeding duration of 4.6 mo as compared to controls who had an average duration of 5.8 mo[47]. Postulated mechanisms have been suggested to include a protective effect of immunoglobulins and antibacterial proteins in breast milk[37]. In addition, breast milk may accelerate maturation of the GI tract in infants, and may also delay the introduction of cow's milk, a potentially allergenic food[37]. Another population based case-control study examined three cohorts of patients: one with 638 CD patients, one with 653 UC patients, and 600 controls[46]. Specific factors associated with a lower odds ratio of CD and UC included breast-feeding (CD: OR = 0.55; 95%CI: 0.41-0.74; UC: OR = 0.71; 95%CI: 0.52-0.96), and having a vegetable garden during infancy, childhood or adolescence (CD: OR = 0.52; 95%CI: 0.36-0.76; UC: OR = 0.65; 95%CI: 0.45-0.94)[46]. In addition, those living in the countryside had a lower odds ratio of having CD (OR = 0.64; 95%CI: 0.46-0.89).

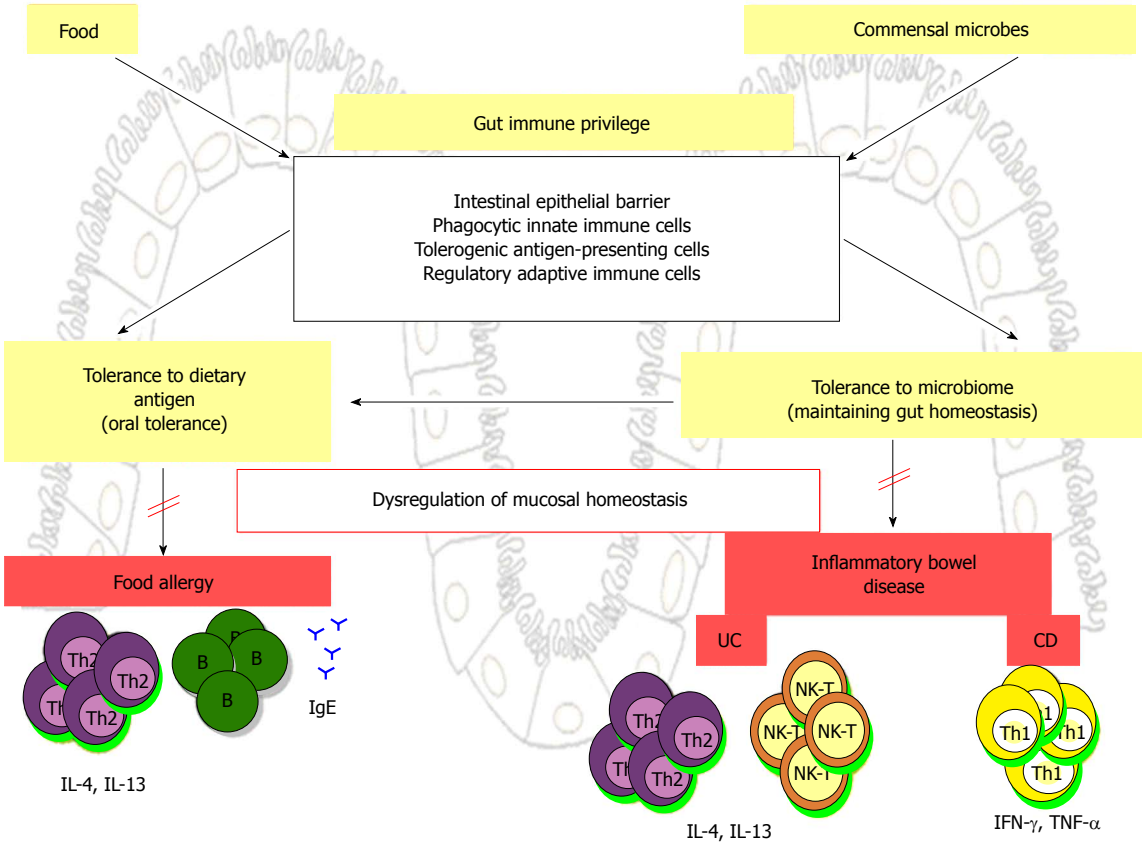

Figure 2 Immune privilege in the gut consists of tolerance to dietary antigens and to commensal microbes[49]. Reproduced with permission from reference Iweala *et al*[49]. CD: Crohn's disease; UC: Ulcerative colitis; TNF-α: Tumor necrosis factor-α; IL: Interleukin; IFN: Interferon.

The duration of breastfeeding was also significant in decreasing both IBD and UC, with those having 0-2 mo of exposure having no protection, as compared with those having 3 or more mo[46].

Relation between pathophysiology of food insensitivity and IBD

While 20%-30% of the general population have undesirable reactions to food, only 10%-25% of these are actual food allergies[48]. The gastrointestinal mucosa is however predisposed to develop allergic reactions, since the tissue in the GI tract is exposed to various food and bacterial allergens in addition to containing all cells required to develop allergic reactions, such as eosinophils, mast cells, and lymphocytes[48].

Proteins in food usually act as the primary foreign antigens that trigger most allergic reactions in the gastrointestinal tract[49]. The border between intra and extraluminal sites in the intestine plays a vital role in preventing inappropriate inflammation or allergic disease in the gut[49,50]. The gut barrier works in at least five different ways to prevent such diseases. These include (Figure 2): (1) a physical barrier preventing foreign or microbial invasion; (2) the presence of specialized immune cells, including macrophages, to phagocytize microbes and other proinflammatory or allergic antigens; (3) release of IgA, which sequesters microbes away from the gut in the intraluminal space; (4) promotion of antigen presenting cells which upregulate immune tolerance; and (5) expansion of regulatory T cells which dampens the immune response[49]. Chronic inflammation in the gut may act in an ongoing fashion with increasing inflammation leading to additional damage to the physical barrier of the gut, leading in turn to more proinflammatory antigens which can then pass into the extraluminal space[51].

A disruption of one of these five regulatory barriers may contribute to inflammation and/or allergic responses. Mast cells and eosinophils also play a critical role in modulating intestinal allergic reactions and have also been found to be stimulated in IBD and eosinophilic gastroenteritis[52].

In addition, the main inflammatory mechanisms of IBD and of the pathophysiology of intestinal allergic phenomena are related concepts. In particular, the Th2-like response seen in ulcerative colitis is driven by NK-T cells activated by glycolipid and CD1 on epithelial cells, which facilitates their production of proinflammatory cytokines including IL-13 and IL-5[53]. Interestingly, this process does not result in the production of IL-4. Upregulation and maladaptive Th2 responses have been implicated in chronic rhinosinusitis[54]. In addition, *Staphylococcus aureus* (*S. aureus*), a common colonizer of the nasal tract, has been shown to upregulate IL-5 and IL-13 in nasopharyngeal lymphocytes[54].

Additionally, there is also maladaptive activity of T regulatory T cells (Tregs) both in IBD and in food intolerance[49]. Adoptive transfer of Tregs has been shown to prevent intolerance to the food antigen OVA[55]. In addition it has been found that TLR4 signaling is critical for

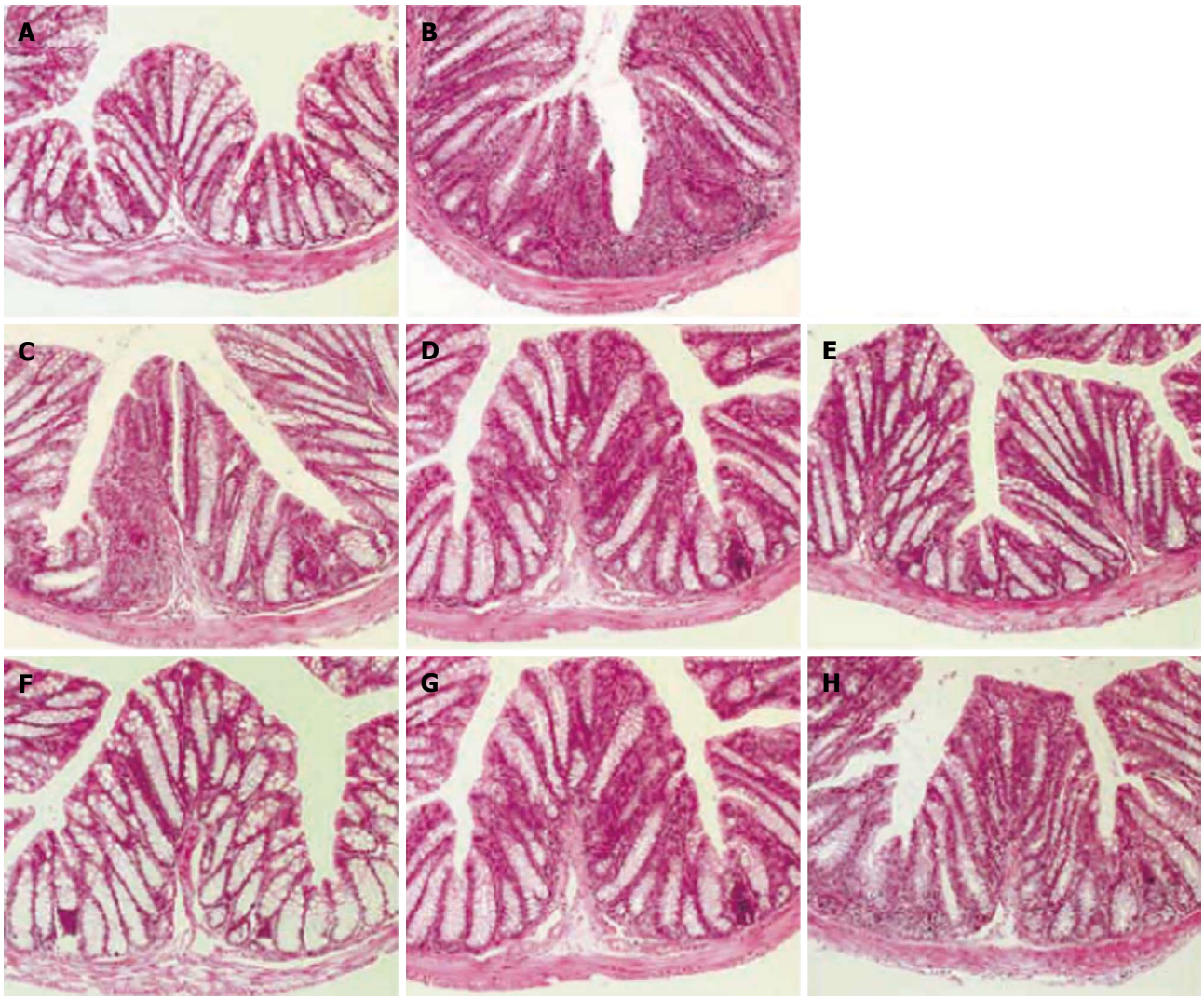

Figure 3 Histologic analysis of the colon in C57BL/6 mice. A: Normal architecture of the colonic mucosa from mice treated with 50% ethanol alone; B: Erosions of the epithelium, distortion of crypts, loss of goblet cells, and massive mononuclear cell infiltration in lamina propria in mice after administration of trinitrobenzene (TNBS); C-E: TNBS-induced colitis is dose-dependently improved by curcumin. Mice were treated with 0.5% (C), 2.0% (D), or 5.0% (E) curcumin just after administration of TNBS; F-H: Mice were treated with 2% curcumin in preventive mode (F), early therapeutic mode (G), or late therapeutic mode (H) (original magnification × 50). Reproduced with permission from reference Sugimoto *et al*[66].

Treg suppression of responses to food antigens (regulating food tolerance) and also to commensal bacteria (in prevention of colitis)[49].

Common treatments for food insensitivity and IBD

Alterations in diet have been used as a treatment for IBD. The use of enteral feeding has been shown to be beneficial, with a majority of studies showing a remission rate of over 60%[56]. Liquid feeding is thought to be helpful because it leads to more rapid transit time for feeds, induces partial bowel rest, as well as alters the fecal flora present[57]. Tube feeding with an enteral elemental liquid formula cannot be used long-term however, as most patients do not tolerate this, and IBD typically relapses after discontinuation of the diet[57]. However, the use of polymeric drinks may be better tolerated and shows similar effectiveness in this population[57].

The use of elimination diets has also been studied. These typically involve the use of a food diary, with elimination of symptom provoking foods, or the use of a basic diet with reintroduction of potentially problematic foods one food type at a time[58]. This approach was shown to be beneficial in inducing remission in six of nine studies[58]. Referral to a nutritionist may be of significant benefit[58].

Other potential therapies in the treatment of IBD include the use of polyunsaturated fatty acids (PUFA). These acids, including omega-3 fatty acids, decrease inflammation as their derivatives, including eicosapentaenoic acid, and leukotriene derivatives, downregulate neutrophil trafficking and thereby decrease edema formation[59-61]. Twelve studies with n-3 PUFA, mostly from fish oil, showed benefit in IBD patients, with a decreased need for steroids, diminished disease activity and a lower relapse rate[59].

Several herbal preparations are considered useful by some in the treatment of inflammatory disorders. Lonicera japonica is a Korean traditional treatment, and has been shown to decrease histamine release from mast

cells and inhibit inflammatory pathways, including the NF-κB and AP-1 pathways[62-64]. Lonicera may be an attractive agent for future clinical trials in both IBD and allergic disease[62]. In addition, curcumin and green tea polyphenols have been shown to be potent antioxidants and have anti-inflammatory activity in mice[65-67]. In one study, 35.5% of mice treated with trinitrobenzene (TNBS) died after developing an ulcerative colitis-like disease; however, no mice died in a group preventively given a 2% curcumin solution[66]. In addition, mice given doses of curcumin after TNBS-induced colitis demonstrated histologic improvement of colonic mucosa[66] (Figure 3).

DIFFERENCES BETWEEN EOSINOPHILIC COLITIS AND IBD

Eosinophilic gastrointestinal disorder (EGID) is marked by GI inflammation and intense infiltration of the GI tract with eosinophils seen in the absence of other identified systemic disorders such as malignancy, collagen-vascular disease, IBD, parasitic disease, and medication induced eosinophilia[68]. In 50%-70% of patients with EGID, there is a family history of allergic disorders or a personal history of atopy[48]. Symptoms vary between children and adults: in children, the common symptoms are vomiting and abdominal pain, while in adults common symptoms include food impaction, difficulty swallowing, chest pain and heartburn[68]. Symptoms mimic both irritable bowel syndrome and IBD. Histologically, EGID can be distinguished from GERD; more than 15-20 eosinophils per high power field are seen on biopsy in EGID as opposed to less than 5 in GERD[48,68]. Both elimination and elemental diets have shown promise in the treatment of patients with EGID[48,68].

CONCLUSION

While the immunological underpinnings of both IBD and allergic disease are complex and multifaceted, the degree of overlap between the two disorders is striking. Further studies are warranted to try to help better understand their complex basis and commonality, and there is much to be gained by studying treatments that benefit patients with these illnesses.

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P- Reviewer: Liu ZJ **S- Editor**: Zhai HH **L- Editor**: A
E- Editor: Zhang DN

World Journal of Gastroenterology

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DOI: 10.3748/wjg.v20.i32.11033

World J Gastroenterol 2014 August 28; 20(32): 11033-11053
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (2): Hepatitis C virus

Chronic hepatitis C and liver fibrosis

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Supported by Grant from the Natural Sciences and Engineering Research Council of Canada (NSERC) to Pantopoulos K, No. RGPIN 288283-2011; Gkouvatsos K is recipient of a doctoral fellowship from the Fonds de la Recherche en Santé du Quebéc (FRSQ); Sebastiani G and Pantopoulos K hold Chercheur-Boursier and Chercheur National Career Awards, respectively, from the FRSQ
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Received: January 16, 2014 Revised: April 14, 2014
Accepted: June 13, 2014
Published online: August 28, 2014

Abstract

Chronic infection with hepatitis C virus (HCV) is a leading cause of liver-related morbidity and mortality worldwide and predisposes to liver fibrosis and end-stage liver complications. Liver fibrosis is the excessive accumulation of extracellular matrix proteins, including collagen, and is considered as a wound healing response to chronic liver injury. Its staging is critical for the management and prognosis of chronic hepatitis C (CHC) patients, whose number is expected to rise over the next decades, posing a major health care challenge. This review provides a brief update on HCV epidemiology, summarizes basic mechanistic concepts of HCV-dependent liver fibrogenesis, and discusses methods for assessment of liver fibrosis that are routinely used in clinical practice. Liver biopsy was until recently considered as the gold standard to diagnose and stage liver fibrosis. However, its invasiveness and drawbacks led to the development of non-invasive methods, which include serum biomarkers, transient elastography and combination algorithms. Clinical studies with CHC patients demonstrated that non-invasive methods are in most cases accurate for diagnosis and for monitoring liver disease complications. Moreover, they have a high prognostic value and are cost-effective. Non-invasive methods for assessment of liver fibrosis are gradually being incorporated into new guidelines and are becoming standard of care, which significantly reduces the need for liver biopsy.

Key words: Hepatitis C virus; Liver fibrosis; Cirrhosis; Biopsy; Fibroscan

Core tip: Chronic hepatitis C is a leading cause of liver-related morbidity and mortality and predisposes to liver fibrosis, the excessive accumulation of extracellular matrix proteins. The staging of liver fibrosis is critical for the management and prognosis of patients. This review provides an update on hepatitis C virus (HCV) epidemiology, summarizes basic mechanisms of HCV-dependent liver fibrogenesis, and discusses common methods for assessment of liver fibrosis. While liver biopsy was until recently considered as the gold standard, novel non-invasive methods, including serum biomarkers, transient elastography and combination algorithms, are gradually being incorporated into new guidelines and are becoming standard of care.

Sebastiani G, Gkouvatsos K, Pantopoulos K. Chronic hepatitis C and liver fibrosis. *World J Gastroenterol* 2014; 20(32): 11033-11053 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11033.htm DOI: http://dx.doi.

org/10.3748/wjg.v20.i32.11033

BURDEN OF CHRONIC HEPATITIS C: THE SCREENING DILEMMA

Chronic hepatitis C (CHC) is caused by infection with hepatitis C virus (HCV) and constitutes a major public health concern, affecting around 200 millions people worldwide[1]. It is the leading cause of hepatocellular carcinoma (HCC) and the main indication for liver transplantation in Western countries. Although some data indicated that HCV does not increase all-cause mortality[2], other studies postulated that CHC could reduce life expectancy by 8 to 12 years[3,4]. Thus, HCV was reported to cause more than 86000 deaths in Europe in 2002[5]. The mortality and morbidity attributable to CHC is expected to increase dramatically over the next 50 years, considering that the rate of new HCV infections dropped significantly only after 1989[6]. Markov model analysis suggested that by 2030, 30% of deaths due to HCV-related complications would be preventable by increasing 50% of the patients receiving treatment with interferon/ribavirin therapy[7]. With the development in new anti-HCV agents, including NS3/4A, NS5A and NS5B inhibitors, higher success rates for treatment are anticipated, even for patients with cirrhosis or post transplantation.

The acute infection with HCV frequently does not resolve spontaneously. Approximately 80% of the infected individuals become chronic carriers and may progress to severe liver disease. Based on the natural history of CHC it is estimated that 10%-20% of patients will develop liver cirrhosis and 1%-5% will develop HCC within 20-30 years[8]. Once liver cirrhosis is established, HCC develops at a yearly rate of 5%-7%[9]. Importantly, epidemiological studies have shown that most patients are unaware of their positive HCV antibody status[6]. A report commissioned by the Institute of Medicine of the National Academies highlighted shortcomings in care for viral hepatitis, and estimated that up to 75% of patients with CHC remain undiagnosed[10]. Along these lines, the Centers for Disease Control and Prevention (CDC) estimated that although persons born during 1945-1965 comprise approximately 27% of the United States population, they account for 75% of all HCV infections, 73% of HCV-related mortality, and are at greater risk of HCC and end-stage liver complications.

Given the fact that early diagnosis and treatment can prevent liver cirrhosis and HCC, it is reckoned that one-time testing of persons born during 1945-1965 (baby boomers) will prevent more than 120000 deaths in the United States. Based on these epidemiological data and on recent advances in treatment of CHC, the CDC is now recommending a general screening strategy with a one-time testing without prior ascertainment of HCV risk for baby boomers[6]. A recent study showed that broader screening for HCV would likely be cost-effective[11]. Nevertheless, significant reduction of HCV-related morbidity and mortality would also require improved rates of referral, treatment and follow-up[11]. Thus, once patients with CHC are recognized from a broader screening for HCV infection, they have to be offered appropriate clinical care and therapy. In this view, the assessment of liver fibrosis stage is the key event in clinical management of CHC, affecting both disease prognosis and treatment indication[12].

HCV AND LIVER FIBROGENESIS: BASIC CONCEPTS

Elucidating the mechanisms underlying liver fibrogenesis is of paramount importance for management and prevention of end-stage liver disease. Liver fibrosis is defined by the excessive accumulation of extracellular matrix (ECM) proteins such as collagen, laminin, elastin, fibronectin, *etc.*, and is currently considered as a wound healing response to chronic liver injury[13]. HCV infection directly modulates signaling and metabolic pathways by viral proteins. Moreover, it indirectly induces host antiviral immune responses leading to chronic inflammation. Together, these events promote liver fibrogenesis[14]. The hepatic stellate cell (HSC), a vitamin A (retinoid)-storing cell residing in the perisinusoidal space of Disse, is the key fibrogenetic element. Although quiescent in the absence of inflammatory stimuli, HSCs are activated in response to liver injury and undergo transformation to proliferative, contractile myofibroblasts. Activated HSCs constitute a prevalent source of ECM production[15] and thereby disrupt the equilibrium between deposition and dissolution of ECM proteins, which leads to fibrotic scarring and eventually to liver cirrhosis (Figure 1).

The development of cell culture and animal models that recapitulate main aspects of HCV infection and liver injury has been crucial for understanding the pathogenesis of CHC[16,17]. This involves pathways that are implicated in the initiation and the perpetuation of HSC activation. Initiation of HSC activation is mediated by paracrine stimuli from neighboring cells, reactive oxygen species (ROS), lipopolysaccharide (LPS), or apoptotic bodies. HSCs maintain their activity in response to fibrogenetic, proliferative, chemotactic and inflammatory signaling[14].

Direct HCV-dependent liver fibrogenesis by viral proteins

The HCV contains a positive sense single-stranded RNA that is translated to a large polyprotein precursor. The latter undergoes proteolytic cleavage by viral and host enzymes in order to generate mature structural proteins (core, E1, E2 and p7) and non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B)[18]. These molecules may target multiple cell types, including hepatocytes, monocytes, lymphocytes and various secretory cells[19-21], and thereby modulate cell proliferation, apoptosis, oxidative stress and innate immunity[22].

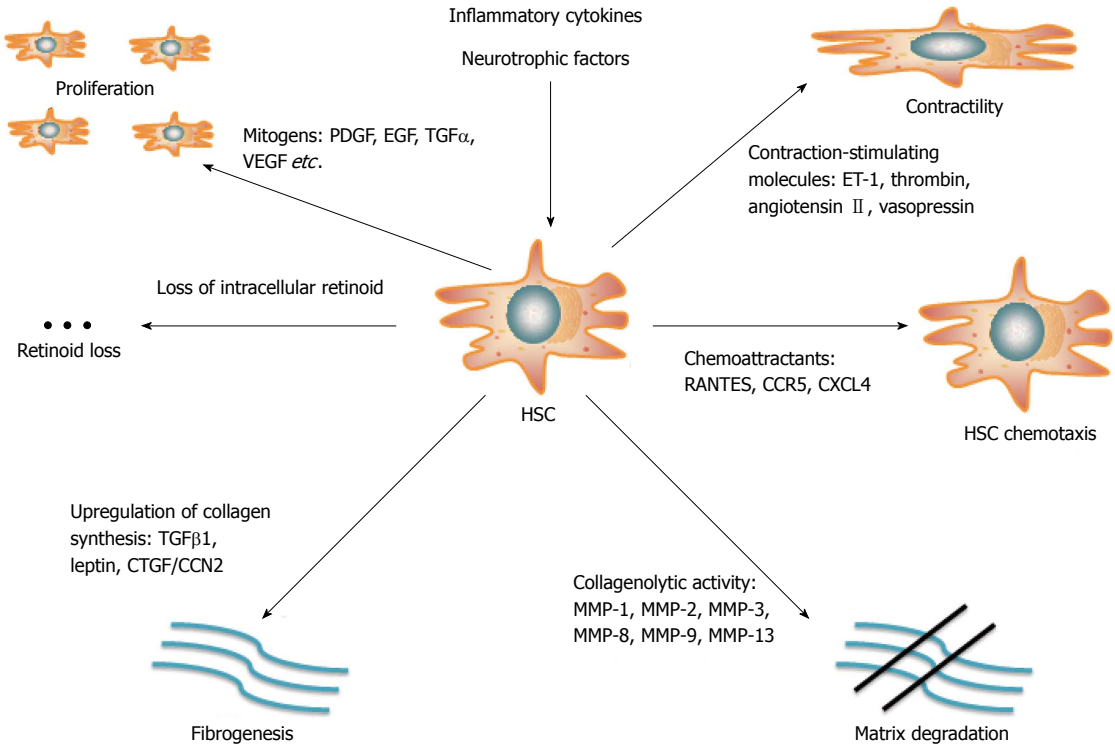

Figure 1 Hepatic stellate cells are retinoid-storing cells that play a key role in liver fibrogenesis. During liver injury, they undergo transformation from a quiescent state to proliferative, contractile myofibroblasts. Activated HSCs are the main source for production of collagen and other ECM proteins. Several molecules and pathways regulate the equilibrium between deposition and degradation of ECM proteins. HSCs: Hepatic stellate cells; ECM: Extracellular matrix; PDGF: Platelet-derived growth factor; EGF: Epidermal growth factor; TGF: Transforming growth factor; VEGF: Vascular endothelial growth factor; CCR5: C-C chemokine receptor 5; MMP-2: Matrix metalloproteinase 2.

Experimental evidence suggests that the HCV core protein, as well as non-structural HCV proteins may directly trigger HSC activation and, thus, the initiation of fibrogenesis. The core protein preferentially activates pro-mitogenic intracellular pathways within HSCs, whereas the NS3 and NS5 proteins specifically stimulate pro-inflammatory pathways *via* NF-κB and JNK[23]. The core and NS3 proteins promote increases in intracellular calcium $[Ca^{2+}]_i$ and ROS levels; the effects of the core protein depend on its binding to the C1q receptor[23]. The induction of osteopontin by calcium and ROS signaling contributes to the epithelial to mesenchymal transition of hepatocytes[24]. The E2 glycoprotein of the HCV envelope is another potential fibrogenetic factor. It promotes the activation of matrix metalloproteinase 2 (MMP-2) upon binding to CD81 of HSCs, which results in degradation of normal ECM in areas with high HCV density, and may lead to infiltration of inflammatory cells[25].

It should also be noted that the core, NS3 and NS5A proteins induce oxidative stress in hepatocytes and monocytes *via* activation of the NADPH oxidase[26-28] and repression of heme oxygenase 1 (HO-1)[29]. In addition, the core and NS3 proteins activate inflammatory pathways *via* Toll-like receptor 2 (TLR2) in monocytes, which modulate innate immunity[30]. Furthermore, studies with HCV replicon models demonstrated the induction of oxidative stress and the activation of transforming growth factor β1 (TGFβ1) and other pro-fibrotic signals in response to HCV replication[31,32].

Indirect HCV-dependent liver fibrogenesis via immune responses and other pathways

The immune response to HCV infection plays a key role in the enhancement of hepatic fibrogenesis. Multiple growth factors, inflammatory cytokines and chemokines may regulate the activation of HSCs and their transformation to myofibroblasts[33]. In particular, the immune-promoted induction of the platelet-derived growth factor (PDGF) and the subsequent mobilization of intracellular calcium elicit mitogenic effects to HSCs[34,35]. Kupffer cell-derived transforming growth factor α (TGFα)[36] and bile acid-induced activation of the epidermal growth factor (EGF) receptor[37] promote the proliferation of HSCs. Moreover, induction of the vascular endothelial growth factor (VEGF) contributes to activation and proliferation of HSCs, as well as to hepatic angiogenesis, rendering this molecule a key element of the fibrogenic process[38].

Next to the proliferative factors, fibrogenic cytokines that promote ECM production are positively regulated in the context of immune responses to HCV infection. TGFβ1 is the most potent pro-fibrotic cytokine, stimulating collagen production *via* Smad signaling[39,40]. Moreover, additional molecules such as the connective tissue growth factor (CTGF/CCN2)[41] and the adipokine leptin[42] promote liver fibrogenesis *via* TGFβ1 signaling. The fibrogenic activity of leptin is partly mediated by TGFβ1 and requires further Kupffer cell-derived stimuli[43]. Leptin also acts as a suppressor of the peroxi-

some proliferator-activated receptor γ (PPARγ), an anti-fibrotic nuclear receptor able to abrogate HSC activation and conserve its quiescence[44].

Chemokines enhance fibrogenesis through chemotaxis of fibrogenic cells and amplification of the inflammatory response. HSCs produce numerous receptors and secret several cytokines[45]; their role in the pathophysiology of fibrogenesis is currently a subject of investigation. Recent evidence suggests that the induction of C-C chemokine ligand 5 (CCL5, also known as RANTES) by the NF-κB signaling pathway promotes chemotactic and mitogenic effects to HSCs *via* its C-C chemokine receptor 5 (CCR5)[46]. Furthermore, platelet-derived chemokine (C-X-C motif) ligand 9 (CXCL9) exhibits anti-fibrotic properties that depend on its receptor CXCR3[47], whereas CXCL4 exerts a pro-fibrotic function[48].

Neurochemical and neurotrophic factors may also enhance the fibrogenetic function of the HSCs. Several cellular pathways of the neuroendocrine system are activated in response to chronic liver injury. Induction of opioid signaling by endogenous opioids stimulates proliferation of HSCs and enhances collagen deposition[49]. Along similar lines, the activation of the CB_1 receptor by HSC-derived cannabinoids[50], the enhancement PDGF signaling in HSCs by serotonin[51] and the activation of HSCs by thyroid hormones[52] promote fibrogenetic pathways.

The direct interaction of HSCs with immune cells, through expression of adhesion molecules, results in bidirectional cellular stimulation and amplification of fibrosis. Tumor necrosis factor α and monocyte chemoattractant protein 1 (MCP-1), along with other pro-inflammatory cytokines are secreted by Kupffer cells in response to NF-κB activation[53]. This results once again in HSCs activation and in secretion of factors that amplify the inflammatory process and perpetuate the macrophage activity, such as the macrophage colony-stimulating factor[54], interleukin 6[55], MCP-1[56] and RANTES[46]. In addition, HSCs express cell adhesion molecules including vascular cell adhesion molecule 1[57] and intracellular adhesion molecule 1[58]. These are involved in further recruitment of inflammatory cells in the site of injury, which enhances the fibrogenetic process. Other cell types implicated in fibrosis progression include lymphocytes[59], macrophages[60] and endothelial cells[61]. Macrophages promote the survival of activated HSCs *via* NF-κB-dependent pathways[62]. By contrast, natural killer cells and T cells from HCV-infected patients promote apoptosis of HSCs and thereby exert anti-fibrotic function[63].

Last but not least, oxidative stress is a key component of hepatic fibrosis[64]. Apoptotic parenchymal cells are being phagocytosed by activated HSCs resulting in activation of the NADPH oxidase[65]. The latter mediates the generation of ROS, which are capable of both initiating and perpetuating fibrosis *via* activation of HSCs, hepatocytes, Kupffer cells and inflammatory cells[66]. This process is further enhanced in the presence of polyunsaturated fatty acids, ethanol and iron. Furthermore, the DNA of apoptotic hepatocytes may interact with HSCs' TLR9 and thus enhance the collagen production and deposition[67].

Mild to moderate hepatic iron overload is a common manifestation of CHC patients. This is largely attributed to misregulation of the iron regulatory hormone hepcidin[68,69], which is transcriptionally inhibited by HCV-induced oxidative stress[70]. Even though iron antagonizes HCV replication by inactivating the viral polymerase NS5B[71,72], hepatic iron accumulation[73], elevated serum ferritin[74] or reduced serum hepcidin levels[75] are associated with progression of liver disease. The hemochromatosis protein HFE, an atypical major histocompatibility complex class I molecule, may also contribute to liver fibrogenesis as an upstream regulator of hepcidin and/or as possible immunological factor[76,77].

IMPACT OF LIVER FIBROSIS ON PROGNOSIS, MANAGEMENT AND SCREENING STRATEGIES

The accumulation of liver fibrosis is a significant incident with major consequences on the pathology development of CHC[78]. It indicates the onset of progressive disease, which may eventually lead to cirrhosis and end-stage liver complications[79]. Patients with absent or mild fibrosis at diagnosis have a relatively low risk (25%-30%) of developing cirrhosis over the next 20 years. Portal and septal fibrosis both cause cirrhosis, albeit with different progression rates (18-20 years for patients with portal fibrosis and 8-10 years for patients with septal fibrosis, respectively)[80]. Thus, the stage of liver fibrosis is critical for clinical management, especially in light of the new screening wave of HCV-infected patients[6].

The clinical management of CHC patients depends on two different stages of liver fibrosis[81]: (1) considerable fibrosis, histologically classified as septal fibrosis (stage F3 by METAVIR), represents a definitive indication to schedule, not defer, antiviral treatment; and (2) cirrhosis (stage F4 by METAVIR) necessitates specific and regular follow-up which should include screening for HCC and esophageal varices. Apart for indication to antiviral treatment, a more advanced liver fibrosis stage should require interventions to control known negative cofactors for disease progression (Table 1). These include life style modifications (diet, weight loss, regular physical exercise), alcohol and drug abstinence, referral to specialists (hepatologist, metabolic clinics, dietician, psychologist), specific medications (statins, insulin-sensitizing agents). Thus, the new screening strategies, which are opening to a large group of persons, the baby boomers, should be associated with diagnostic and therapeutic interventions to all newly identified patients.

LIVER BIOPSY: ALL THAT GLITTERS IS NOT GOLD

For many years the assessment for liver fibrosis has been

Table 1 Factors contributing to fibrosis progression in chronic hepatitis C

Non-modifiable	Modifiable
Duration of HCV infection	High alcohol consumption (≥ 20-50 g/d)
Older age at infection	Insulin resistance
Male sex	Obesity
Presence of baseline fibrosis	Metabolic syndrome
HIV or HBV co-infection	Daily cannabis use
Infection with HCV genotype 3	
Gene polymorphisms involved in iron overload/inflammatory pathways	
Latin ethnicity	

HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; HBV: Hepatitis B virus.

through liver biopsy, which has been considered the gold standard gauge for the direct histological evaluation of the severity of liver disease.

Role of the pathologists in liver biopsy: Errors in samples and reading variability

The representativeness of liver samples obtained through a liver biopsy and the pathologist's experience remain the major determinants of diagnostic accuracy. Inadequate liver biopsy sample can lead to underestimation of liver fibrosis stage[82]. Samples taken from both lobes of the liver in a cohort of CHC patients highlighted in 33.1% of them a difference in the fibrosis stage by at least one grade, and in 14.5% of them underdiagnosis of fibrosis[83]. On single blind percutaneous liver biopsies, cirrhosis was missed in 10%-30% of samples[84-86]. Since liver biopsy involves only a very small part of the whole organ (approximately 1/50000), the diagnosis of fibrosis can be missed, especially in cases where the lesions are not uniformly distributed through the parenchyma.

Misclassification of the stage of liver fibrosis can be reduced by obtaining a specimen of adequate size and quality. It has been suggested by some authors that an adequate sample of the liver should be at least 15 mm in length and ought to contain more than 5 portals[87-89]. By critically evaluating published literature, Guido *et al*[90] concluded that unacceptable methodological limits often flaw liver biopsy results; moreover they proposed sample sizes of 20 mm or more containing at very least 11 complete portal tracts for reliable staging. Analyzing even larger size samples going up to 25 mm in length has been suggested by other authors[91,92]. According to the American Association for the Study of Liver Diseases (AASLD), a liver biopsy sample should contain at least 11 complete portal tracts and be no less than 20 mm in length, while liver fibrosis should be scored by a simple (METAVIR) rather than complex (Ishak) system[93].

There is also a significant degree of inter-/intra-observer variability in the pathologic assessment of liver biopsy samples. The practical knowledge and experience of pathologists demonstrated by a longer medical career, or affiliation within an academic realm, could have a greater influence on the interpretation of the diagnosis, more than the sample size[94]. A pathologist with specific expertise in liver disease should interpret the biopsy, preferably in coordination with the clinician who performed the procedure and is caring for the patient. In the absence of this interaction, diagnostic errors by non-specialist pathologists have been reported in more than 25% of patients[95,96]. If liberal use of second opinions from specialist liver pathologists has been recommended, this may result in increased costs and waiting time.

Recent studies have implied that liver biopsy should not be considered as the gold standard, but rather as the best point of reference for staging liver disease[97,98]. Surrogates in general are evaluated by utilizing the area under the curve (AUC), with liver biopsy as the reference. Mehta and coworkers argued that the ideal surrogate will at no time attain the maximal value (1)[97]. By taking into consideration a spectrum of accuracies of the biopsy plus a spectrum of prevalence of substantial fibrosis, they demonstrated that even under optimal conditions and with a perfect marker, it is not possible to achieve an AUC ≥ 0.90 when assessing substantial fibrosis[97,98].

Invasiveness and cost of liver biopsy from the clinician's perspective

There are definitely advantages in performing liver biopsy since it gives important and direct information relating to fibrosis, necroinflammatory activity, steatosis stage and also hepatic iron deposits, which are recurring histological appearances of CHC and potential comorbidities. However, there are also possible drawbacks for the clinician, such as the invasiveness of the procedure and the cost (Table 2). The most frequent complication (84%) for patients undergoing liver biopsy is pain. Bleeding occurs in 0.01%-0.04% of cases, whereas death is very rarely associated with the procedure (≤ 0.01%). Clinical studies have provided evidence that the rate of complications in percutaneous liver biopsy inversely correlates with the experience of the operator[99,100], but opposite data have also been reported[101].

There is some ongoing debate amongst physicians about liver biopsy and its role in the assessment of fibrosis. A survey with 1177 general practitioners in France showed that up to 59% of patients with CHC refused the procedure due to its invasive nature, and some 22% of the physicians had similar considerations[102]. Liver biopsy was not performed by 29% from 112 American physicians due to following concerns: safety (72.7%), low reimbursement (66.7%), logistical issues (45.4%)[103].

A recent Canadian nationwide survey on patterns of diagnosing liver fibrosis showed that for almost half of the physicians, liver biopsy was the main diagnostic approach. Limitations in access/availability of non-invasive tools and lack of reimbursement represented a significant barrier[104]. A similar survey was earlier performed in France, the country where non-invasive diagnostic methods of liver fibrosis were first marketed, and ap-

Table 2 Comparison of the main characteristics of liver biopsy, serum biomarkers and transient elastography

	Liver biopsy	Serum biomarkers	Transient elastography
Advantages	Direct assessment of liver fibrosis Stage by stage fibrosis classification Evaluation of coexisting disorders (inflammation, steatosis, iron overload)	Immediate result Fast (one time blood sample) Patient friendly	Immediate result Duration of examination 5 min Operator and patient friendly
Limitations	Complications (pain, bleeding) Sampling error, intra-observer and inter-observer variability Hospitalization (day hospital) often required Cost Delayed result (2-4 wk)	Cost (unitary cost per patient for patented tests) High rates of unclassified patients (APRI, Fib-4, Forns' index, Lok index) Lower performance for diagnosis of significant fibrosis Unable to discriminate between intermediate stages of fibrosis	Cost (one time per machine) Failure in 5% of cases (25% in obese patients) Unreliable results in 15% of cases (obesity, ascites, limited operator experience) Lower performance for diagnosis of significant fibrosis Unable to discriminate between intermediate stages of fibrosis
Contraindications	Absolute: uncooperative patient, severe coagulopathy, extrahepatic biliary obstruction Relative: ascites, morbid obesity, possible vascular lesions, amyloidosis	None	Pacemaker, pregnancy
Risk factors for error	Biopsy sample < 2 cm in length, containing < 10 complete portal tracts; inexperienced pathologist	Autoimmune thrombocytopenia (APRI); Gilbert's sydrome, extrahepatic cholestasis, hemolytic anemia (Fibrotest)	Transaminases flares; acute viral hepatitis; non-fasting patient; vascular hepatic congestion; extrahepatic cholestasis; IQR ⩾ 30%

APRI: Aspartate aminotransferase to platelet ratio index; IQR: Interquartile range.

Table 3 Role of liver biopsy and non-invasive tools across the international guidelines

Ref.	Threshold for definitive indication to antiviral therapy	Recommended methods for liver fibrosis staging	Can non-invasive methods replace liver biopsy?
APASL[109], 2007	F1	Liver biopsy	No
AASLD[190], 2009	F2	Liver biopsy, serum biomarkers, transient elastography	No
EASL[81], 2014	F2	Liver biopsy, serum biomarkers, transient elastography	Yes
CASL[111], 2012	None	Liver biopsy, serum biomarkers, transient elastography	Yes

propriate reimbursement policies are being implemented since 2007. Interestingly, only 4% of physicians that responded, routinely requested liver biopsy[105]. A survey among Italian hepatologists uncovered discrepancies between them on how and when to perform liver biopsy in CHC patients[106].

Cost is a major issue for implementation of liver biopsy in clinical practice, especially in light of the recent broader screening strategies for hepatitis C. In the United States the cost is currently $1032 and can increase up to $2745 if complications occur during and after the procedure[107]. In Canada, the mean cost of a complicated liver biopsy requiring hospitalization is $4579[108].

Liver biopsy and non-invasive tools for assessment of liver fibrosis across guidelines

Given the drawbacks of liver biopsy, non-invasive tools for assessment of liver fibrosis have attracted the attention of hepatologists. Table 3 compares guidelines in terms of recommendations for liver biopsy and/or non-invasive tools for the staging of liver fibrosis in HCV-infected patients. Overall, in spite of a previous consensus that a stage of liver fibrosis of at least F2 represents a definitive indication for antiviral therapy, recent guidelines recommend that there should be no threshold precluding patients from antiviral treatment. The Asian Pacific Association for the Study of the Liver (APASL), recommends treatment for patients with a histological score of F1 or above[109]. HCV patients with viral genotypes 1-3 can be treated regardless of the stage of the disease. It is not compulsory for patients infected with HCV genotypes 2 or 3 to have a liver biopsy in order to start therapy. However, obtaining a liver biopsy before starting therapy could offer prognostic information. At the time the APASL guidelines were issued, non-invasive methods were not recommended.

AASLD guidelines state that in CHC, liver biopsy should be considered if the patient and the health care provider wish to know the fibrosis stage to enable an informed decision on treatment options and/or to predict possible outcomes. A liver biopsy may be unnecessary in persons infected with HCV genotypes 2 and 3, since more than 80% of them achieve a sustained virological response (SVR). There is, nevertheless, an ongoing argument on whether CHC patients with HCV genotype 1 warrant a biopsy because of their lower SVR rates.

Likewise, the need for liver biopsy in CHC patients with less common HCV genotypes (4-6) is unclear. At present there are accessible non-invasive tools, which might be useful in determining the absence or presence of advanced fibrosis; however they should not take the place of liver biopsy in routine clinical care practices.

More up-to-date guidelines on management of specific chronic liver diseases, give a different perspective. Thus, according to the European Association for the Study of the Liver (EASL), although liver biopsy is still the gold standard of reference in CHC, non-invasive methods may also be used instead[110]. Similarly, the guidelines of the Canadian Association for the Study of the Liver (CASL) state that acceptable methods to stage liver fibrosis include liver biopsy, Fibroscan® and serum biomarkers[111]. Moreover, the CASL guidelines state clearly that if F2 is a threshold for definitive candidacy to antiviral therapy, no threshold of fibrosis should preclude a patient with CHC from treatment. Overall, the diagnostic value of liver biopsy and non-invasive methods for assessment of liver fibrosis has progressively evolved across the guidelines. In the most recent ones, a clear cut-off for indication to antiviral therapy is no longer recommended. Moreover, we witnessed an evolution in the strength of recommendation of liver biopsy *vs* non-invasive fibrosis assessment tools, with the recent guidelines being indifferent.

NON-INVASIVE ASSESSMENT OF LIVER FIBROSIS: EPIDEMIOLOGICAL AND CLINICAL RATIONALE

The CDC guidelines recommend a onetime screening test for HCV infection in baby boomers, meaning that a new wave of identified chronic carriers will soon present in the panorama of HCV epidemiology. Once these new patients are identified, appropriate management should be offered. Liver fibrosis staging is the single most important factor impacting on the natural history of CHC. It is critical for prognosis and expedited initiation of treatment. However, it is impractical and immensely expensive to stage fibrosis through liver biopsy in all affected persons. Nowadays, this procedure should be thought of as a diagnostic funnel for large-scale screening of liver fibrosis in HCV infection. Consequently, non-invasive tools are absolutely necessary in order to restrict biopsies. In general, non-invasive methods can be divided into two main classes: the serum biomarkers, based on a biological approach; and methods based on a physical approach, including transient elastography, acoustic radiation force impulse imaging, magnetic resonance elastography. Any non-invasive method should ideally fulfill certain characteristics: it should be simple, accessible, easy interpretable, highly accurate, liver-specific, and satisfactorily validated.

The concept of validation is critical and encompasses a number of features that the ideal serum biomarker should fulfill. First, a non-invasive method should demonstrate a good diagnostic accuracy. Specifically, an expensive and patented tool should demonstrate a clear advantage in terms of diagnostic accuracy when compared to simple and economic ones. Second, there should be a sufficient number of validation studies from independent researchers. Third, specific etiology-validation of the non-invasive methods should be provided considering that each etiology of chronic liver disease presents with specific pathogenesis, natural history and associated comorbidities. For example, when considering CHC and chronic hepatitis B (CHB), the former has specific associated comorbidities, such as steatosis and diabetes, the latter is characterized by a more vigorous necroinflammation[112]. Thus, a non-invasive tool developed in the setting of CHB should be specifically validated in CHC patients. Fourth, a careful evaluation of the risk factors for error and failure of a non-invasive tool should be carried out for adequate interpretation in clinical practice. Fifth, serum biomarkers should be specifically validated in special HCV-infected populations, such as patients co-infected with human immunodeficiency virus. Finally, when dealing with serum biomarkers, particularly the patented ones, analytic conditions, such as standardization of reagents and analyzers according to manufacturer's recommendations, should be taken into account. An overview of the non-invasive diagnostic tools for liver fibrosis and their main validation features is shown in Table 4.

SERUM BIOMARKERS FOR ASSESSMENT OF LIVER FIBROSIS

There are direct and indirect serum biomarkers for assessment of liver fibrosis. The former are fragments of compounds of the liver matrix; for instance, hyaluronan, collagen synthesis or degradation products, and regulators of fibrogenetic mechanisms. The latter are biochemical parameters that can be calculated from routine peripheral blood tests. Calculations use liver-derived molecules, such as clotting factors, bilirubin, cholesterol, albumin and transaminases. Direct biomarkers mirror the metabolism of liver ECM and can be potentially utilized to assess the dynamics of liver fibrogenesis. However, they may not be routinely provided in every hospital setting, limiting their clinical use. Indirect biomarkers correlate with liver fibrosis stage. Tables 4 and 5 provide an overview of the performance of the most proven biomarkers in CHC.

Direct biomarkers of liver fibrosis

The most common direct markers investigated for liver fibrosis in CHC include laminin, hyaluronan, procollagen Ⅲ, collagen type Ⅳ, YKL-40, MMPs and their inhibitors (Tables 4 and 5). Hyaluronan is a glycosaminoglycan synthesized by HSCs and degraded in the liver sinusoidal cells[113]. In a study of 326 CHC patients, the AUC for significant fibrosis and cirrhosis were 0.86 and 0.92, respectively, and the cut off level was 110 μg/L[113]. Nevertheless, a different cohort study involving over 400 patients

Table 4 Main validation features among the non-invasive methods for liver fibrosis diagnosis

Ref.	Parameters	Independent validation studies	Etiology-validation studies	Characterization of risk factors for error	Validation in special HCV populations
AAR[138]	AST, ALT	+	+	+	+
APRI[142]	AST, platelets	+	+	+	+
ELF[131]	Age, TIMP-1, hyaluronan, procollagen type Ⅲ	+/-	+	+	-
Fib-4[145]	Age, ALT, AST, platelets	+	+	+	+
Fibrometer®[122]	Platelets, prothrombin index, AST, α2-macroglobulin, hyaluronan, urea, age	+/-	+	+	+
Fibroscan®[167]	Liver stiffness measurement	+	+	+	+
Fibrospect®[132]	Hyaluronan, TIMP-1, α2-macroglobulin	+/-	-	-	-
Fibrotest-Fibrosure®[132]	γGT, total bilirubin, haptoglobin, α2-macroglobulin, apolipo-protein A1, age, gender	+	+	+	+
Forns' index[144]	Age, γGT, cholesterol, platelets	+	+	+	+
Hepascore[129]	Age, gender, bilirubin, γGT, hyaluronan, α2-macroglobulin	+/-	+	-	+
Hyaluronan	Hyaluronic acid	+	+	+	+
Lok index[191]	AST, ALT, platelets	-	-	+	-

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; AAR: AST-to-ALT ratio; APRI: AST-to-platelet ratio index; AP: Age-to-platelet ratio; HCV: Hepatitis C virus; TIMP-1: Tissue inhibitors of metalloproteinases-1.

Table 5 Diagnostic performance of serum biomarkers in chronic hepatitis C

Index	⩾ F2/F4						
	AUC	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	LR+	LR-
Hyaluronan[113-115,119,128]	0.73-0.86/ 0.89-0.92	64.5-75/ 79.2-100	81.0-91.2/ 80.0-89.4	44.0-86.3/ 63.0-100	78.5-93/ 99.0-100	3.94-7.32/ 5.00-7.47	0.30-0.38/ 0.00-0.23
Fibrometer[122,124]	0.85-0.89/ 0.91	80.5-89/ 94.1	84.1-89.9/ 87.6	82.0-86.3/ 68	77.6-82.5/ 94.7	5.56-7.97/ 7.46	0.13-0.21/ 0.06
FibroSpect[122,126-128]	0.82-0.87/ NA	71.8-93.0/ NA	66.0-73.9/ NA	60.9-82.6/ NA	77.7-94/ NA	2.73-2.75/ NA	0.10-0.24/ NA
Hepascore[124,129,130]	0.79-0.85/ 0.85-0.94	53.08-82/ 71.0-76.5	65.0-92.0/ 84.0-89.8	70-88/ 64.9	63.5-78/ 89.6-98	2.34-6.62/ 4.78-6.96	0.27-0.51/ 0.27-0.32
ELF score[122,131]	0.80/ NA	90/ NA	31/ NA	27.5/ NA	92/ NA	1.30/ NA	0.32/ NA
AAR[137,192]	NA/ 0.51-0.83	NA/ 46.7-78.0	NA/ 95.9-100	NA/ 73.7-100	NA/ 80.7-89	NA/ 19.02	NA/ 0.22-0.43
APRI[122,124,133,137,142,192-194]	0.69-0.88/ 0.61-0.94	41-91/ 57-89	47-95/ 75-93	61-88/ 38-57	64-86/ 93-98	1.71-8.20/ 3.56-8.14	0.19-0.62/ 0.10-0.46
Lok Index[137,191]	NA/ 0.78-0.81	NA/ 37-92	NA/ 30-94	NA/ 32-75	NA/ 84-91	NA/ 1.31-6.16	NA/ 0.26-0.67
Forns' Index[122,124,133,144,192,193]	0.60-0.86/ NA	79.8-94/ NA	61.2-95.0/ NA	66-94.7/ NA	63.8-96/ NA	2.42-15.96/ NA	0.09-0.21/ NA
Fib-4[145]	0.82-0.89/ 0.79-0.91	37.6-74.3/ NA	80.1-98.2/ NA	82.1/ NA	94.7/ NA	3.73-20.77/ NA	0.32-0.63/ NA
Fibrotest[122,124,132,133,135]	0.74-0.87/ 0.71-0.87	65-77/ 50-87	72-91/ 70-92.9	76-80/ 57.9-93	66.7-81/ 44-90.5	2.75-7.22/ 2.9-7.04	0.31-0.38/ 0.17-0.53

AUC: Area under the curve; NA: Not available; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; AAR: AST-to-ALT ratio; APRI: AST-to-platelet ratio index.

reported an AUC of only 0.73 for significant fibrosis[114]; cirrhosis was excluded with 100% negative predictive value (NPV), a cut-off of 50 μg/L and an AUC of 0.97. In yet another study with 486 patients, hyaluronan values of < 60 μg/L were used to exclude cirrhosis with a NPV of 99%[115]. Type Ⅳ collagen showed an AUC of 0.83 for the diagnosis of significant fibrosis[116]. Comparison of the diagnostic performance of hyaluronan and type Ⅳ collagen revealed superiority of the former as a marker in CHC[117].

Laminin is a non-collagenous glycoprotein synthesized by HSCs and deposited in the liver basement membrane. The diagnostic value of laminin is not as high as those of hyaluronan and type Ⅳ collagen[118]. Thus, a study involving 243 chronic liver disease patients reported a 77% accuracy for laminin for detecting significant liver fibrosis among a CHC subgroup[119]. MMP-2 and tissue inhibitors of MMP-1 and -2 (TIMP-1 and -2) have also

demonstrated some diagnostic potential to detect liver fibrosis in CHC[120].

YKL-40 is a glycoprotein that is member of the chitinase family. It is strongly expressed in human cartilage and liver, and it is involved in the fibrogenetic process. In 109 CHC patients, it showed a discrete performance for significant liver fibrosis AUC 0.81, specificity of 81% and sensitivity of 78%. However, its accuracy for the prediction of liver cirrhosis was lower, with the AUC at 0.795 A possible diagnostic value of procollagen Ⅲ assessment has also been evaluated; however, it was found to be inferior compared to type Ⅳ collagen and hyaluronan[113,121].

Direct markers have also been proposed as combination panels for increasing the diagnostic performance of the single parameter. Fibrometer® is a patented test combining age, platelets, hyaluronan, AST, prothrombin index, urea and α2-macroglobulin. In CHC patients, AUC values were reported to be between 0.85-0.89 for significant liver fibrosis and 0.91 for liver cirrhosis[122-124]. Fibrospect® is a combination of hyaluronan, TIMP-1 and α2-macroglobulin that showed an AUC of 0.82-0.87 for significant fibrosis[125-127]. A comparative study investigated the diagnostic performance of Fibrospect®, hyaluronan and YK-40 for significant fibrosis in CHC[128]. Interestingly, the recorded Fibrospect® AUC was 0.66, while that of hyaluronan was 0.76. Hepascore® is another patented test, combining age, gender, hyaluronan, bilirubin, γGT, and α2-marcoglobulin. In CHC patients, AUC values of Hepascore® were 0.79-0.85 for diagnosis of significant fibrosis, and 0.89-0.94 for diagnosis of cirrhosis, which indicates an excellent performance[124,129,130]. The panel of direct non-invasive markers proposed by the European liver fibrosis study group includes, hyaluronan, TIMP-1, type Ⅲ collagen and age. In a cohort study involving more than one thousand patients with chronic liver disease, the panel detected significant liver fibrosis with an AUC of 0.77 in the CHC subgroup[131].

Among the patented panels combining parameters for diagnosis of liver fibrosis, Fibrotest-Fibrosure® is the most validated. The parameters included in its formula are γGT, total bilirubin, haptoglobin, α2-macroglobulin, apolipoprotein A1, age and gender[132]. Risk factors for error of this test include elevation of bilirubin levels unrelated to fibrosis (for example due to cholestatic or Gilbert syndromes), reduction of haptoglobin related to hemolysis, elevation of haptoglobin and α2-macroglobulin due to non-hepatic inflammation. The number of patients that have been included in independent studies is more than 5000. The AUC values range between 0.74-0.87 for significant fibrosis and 0.71-0.87 for cirrhosis[89,132-134]. A systematic review including 9 studies for a total number of 1679 CHC patients concluded that Fibrotest-Fibrosure® is excellent for its diagnostic accuracy in cirrhosis but not in early stages of fibrosis[135].

Indirect biomarkers of liver fibrosis

Non-invasive indirect biomarkers for liver fibrosis comprise serum parameters and their combination panels, such as platelets, transaminases, and albumin. Platelet count showed a discrete performance in ruling-out cirrhosis with a cut-off value of 150×10^9/L, with 84% to 95% NPV[119,136,137]. The prothrombin index, based on prothrombin time, showed a NPV ranging from 82% to 91% to rule-out cirrhosis[119,137]. However, these simple and inexpensive markers do not provide a classification of significant liver fibrosis.

One of the most adopted indirect biomarkers is the AST to ALT ratio (AAR), which is widely used for the staging of liver fibrosis in CHC patients. The normal value is < 0.8. An increase of AAR reflects a progressive liver functional impairment, while a ratio ⩾ 1 is indicative of cirrhosis[138]. AAR distinguished cirrhotic patients from non-cirrhotic with 60%-83.6% accuracy, 31.5%-81.3% and 53%-100% specificity[138-141]. Its performance has been variable in difference studies, and the AUC ranged between 0.51-0.83. This test is easy to perform in the daily clinical setting and it comes with no cost; however, a major limitation is that it cannot diagnose significant fibrosis, while values may be affected in case of alcohol consumption[119].

The AST to platelet ratio index (APRI) is another simple score proposed for the classification of both significant fibrosis and cirrhosis. The APRI is calculated by using AST and platelet count, which makes it easily accessible to the clinician at virtually no cost[142]. It is a useful tool to manifest or exclude significant liver fibrosis (cut-off 0.5-1.5) and liver cirrhosis (cut-off 1-2). However, in a substantial number of patients (30%-50%) APRI values are within an intermediate area and thus classification is unreliable. Nonetheless, to date APRI remains one of the most validated non-invasive biomarkers for liver fibrosis, and among the most referenced by guidelines[89]. In the initial study, APRI demonstrated a high precision for the prediction of significant fibrosis (AUC 0.88) and cirrhosis (AUC 0.94)[142]. Subsequent studies nevertheless indicated an irregular performance with AUC for significant fibrosis ranging between 0.69-0.88 and for cirrhosis between 0.61-0.94[89,133]. This variability could be partially explained by different cut-off values chosen in each study and by population heterogeneity. A recent meta-analysis of 40 studies, which included 8739 patients with CHC, concluded that APRI can be used in clinical practice for the confirmation of severe fibrosis/cirrhosis when other clinical signs and examination are non-decisive[143]. Moreover, since it is cheap and simple, it should be considered a reference test against which other non-invasive methods should illustrate improved precision and cost-effectiveness. Moreover, APRI is still the first choice for CHC patients to identify fibrosis in regions with limited healthcare resources.

The Lok index is a modification of APRI that combines platelet count, INR and AAR102. Cut-off values of 0.2 or 0.5 are used to rule-out or rule-in cirrhosis, respectively. Nevertheless, the Lok index is unreliable in detecting significant fibrosis. To this end, Forns *et al*[144] developed a simple panel based on clinical variables rou-

Table 6 Cut-off values, performance and number of patients per study of Fibroscan®

Ref.	Cut-off for ⩾ F2 (kPa)	Cut-off for F4 (kPa)	AUC for ⩾ F2	AUC for F4	Number of patients included
Sandrin *et al*[147], 2003	7.6	14.4	0.88	0.99	106
Castéra *et al*[167], 2005	7.1	12.5	0.83	0.95	183
Ziol *et al*[195], 2005	8.7	14.5	0.79	0.97	327
Kettaneh *et al*[196], 2007	6.8	17.6	0.79	0.91	935
Arena *et al*[197], 2008	7.8	14.8	0.91	0.98	150
Cross *et al*[198], 2010	8.9	10.1	0.89	0.97	187
Degos *et al*[199], 2010	5.2	12.9	0.75	0.90	913

AUC: Area under the curve.

tinely recorded: age, γGT, platelet count and cholesterol levels. The Forns' index utilizes cut-off values of 4.2 or 6.9 to rule-out or rule-in significant fibrosis, respectively, while intermediate values cannot be classified. A study involving 476 CHC patients revealed a high diagnostic performance of the Forns' index for the detection of significant fibrosis, with an AUC of 0.81-0.86[144]. Remarkably, the low cut-off value of 4.2 had a NPV of 96% in excluding significant liver fibrosis. Conversely, the high cut-off value of 6.9 had a positive predictive value of only 66% in manifesting significant fibrosis. Further studies uncovered a slightly decreased performance of Forns' index, with AUC 0.76-0.79[124,133]. The major limitation of the Forns' index is that it does not offer conclusive information regarding cirrhosis, while it leaves a high number of cases unclassified.

Fib-4 is another index combining simple biomarkers and is based on age, platelet count, AST, and ALT[145]. Fib-4 uses cut-off values of 1.45 or 3.25 to rule-out or rule-in significant fibrosis, respectively. In a study involving 529 CHC patients, Fib-4 enabled the correct identification of cases with severe fibrosis and cirrhosis, with AUC 0.85[145]. Similar conclusions were reached by other studies[146]. Nonetheless, overall Fib-4 does not offer sufficient clues about cirrhosis and consistently leaves several cases unclassified. On the other hand, it is simple and cheap, and has been validated in a number of studies.

NON-INVASIVE ASSESSMENT OF LIVER FIBROSIS BY TRANSIENT ELASTOGRAPHY

The measurement of liver stiffness by transient elastography offers an accredited non-invasive method for the assessment of liver fibrosis[147]. It is performed by using Fibroscan® (Echosens, Paris), a device composed of an ultrasound transducer probe that is mounted on the axis of a vibrator. The transducer transmits vibrations of mild amplitude and low frequency. This generates an elastic shear wave, which disseminates through the underlying tissue. Dissemination of the shear wave is monitored by pulse-echo ultrasound acquisition. Its velocity directly correlates to tissue: the faster the shear wave disseminates the stiffer the tissue. Liver stiffness is measured by Fibroscan® in a volume that is approximately a cylinder 1 cm wide and 4 cm long, between 2.5 and 6.5 cm below the skin. This volume is substantially bigger (at least 100 times) than a typical biopsy sample.

The Fibroscan® examination is painless, fast (performed in less than 5 min), and easy to use. It is performed on a patient who is lying flat on his/her back, with the right arm tucked behind the head. The probe transducer is placed on the patient's skin, in-between the rib bones at the same level as the right lobe of the liver that would be used to obtain a biopsy sample. The operator needs to acquire 10 valid measurements and then the Fibroscan® software calculates the median value. Success of each measurement is determined by the software itself. Liver stiffness ranges between 2.5-75 kPa. Fibroscan® cut-off values between 5.2-8.9 kPa are consistent with significant fibrosis, while values between 10.1-17.6 kPa indicate cirrhosis[79,148]. Main features on Fibroscan® studies in CHC patients are summarized in Table 6.

Overall, the accuracy of transient elastography is comparable to that of patented serum biomarkers that are used for assessment of significant liver fibrosis, with AUC < 0.80. However, transient elastography shows excellent performance for the diagnosis of cirrhosis since the AUC was ⩾ 0.90 in all reported studies[79]. Meta-analysis data indicate that the Fibroscan® examination alone does not provide sufficient information to diagnose significant liver fibrosis. Instead, Fibroscan® may be used together with an algorithm combining non-invasive serum biomarkers[149]. On the other hand, the meta-analysis validated the excellent accuracy of transient elastography in the diagnosis of liver cirrhosis when other examinations and clinical signs are inconclusive. It should be noted that the French *Haute Autorité de Santé* recommends the utilization of either Fibroscan®, Fibrotest® or Fibrometer® for first line assessment of liver fibrosis in CHC patients.

Applicability of Fibroscan® in clinical practice

Even though the Fibroscan® examination *per se* is straight forward, the interpretation of the result must be done by an expert clinician, knowledgeable on the clinical background of the individual patient and on the conditions that can influence liver stiffness measurement. Factors that influence the applicability of Fibroscan® in clinical practice can be divided into three categories: (1) risk factors of failure; (2) risk factors of low quality; and (3) risk factors of false positivity.

Risk factors of failure of liver stiffness measurement include obesity, narrow intercostal space and ascites[79]. Failure rates range between 2.4%-9.4%[150,151]. Obesity is a major factor for failure, given its frequency in the general population. A study of 2114 examinations showed that a body mass index (BMI) $\geqslant$ 28 kg/m^2 was the only factor independently associated with failure[152]. On the same line, Wong and colleagues found a failure rate of 2.6% if BMI was < 30 kg/m^2 and 25.5% if BMI was $\geqslant$ 30 kg/m^2[153]. To overcome the high failure rates occurring in obese patients with the Fibroscan® standard probe (M), a new FibroScan® probe (the "XL" probe) has been developed. This utilizes a hypersensitive ultrasonic transducer with a lower frequency, larger vibration amplitude, deeper focal length and higher depth of measurement. Reliable results with the XL probe were obtained in 61% of obese patients in whom the M probe failed[154].

According to the manufacturer, the risk factors of poor quality of a Fibroscan® examination include an interquartile range (IQR) exceeding 30% of the median value, which reflects the variability of the validated measures, and a success rate less than 60%, that is the percentage of valid measurement. Interestingly, a study investigating 254 CHC patients showed that while IQR is indeed a factor of overestimation of liver fibrosis, success rate is not a factor significantly influencing the accuracy of Fibroscan®[151].

A number of conditions can lead to false positivity of Fibroscan® examination. Acute viral hepatitis increases liver stiffness[155,156]. Thus, the necroinflammatory status needs to be taken into consideration, particularly in patients with absent or low-stage liver fibrosis. In relevant studies[155,156], ALT levels correlated with Fibroscan® values. Conversely, another study showed that low AST is a variable associated with discordance between Fibroscan® measurement and liver biopsy for diagnosis of significant fibrosis[157]. The authors concluded that Fibroscan® is influenced by major variations in biochemical activity of liver disease in CHC and that liver stiffness, at low levels of AST, can underestimate fibrosis. For this reason, adjustments for age and AST of the Fibroscan® result may significantly improve accuracy.

In patients with extra-hepatic cholestasis, liver stiffness significantly correlates with bilirubin levels and leads to false positivity of Fibroscan® measurement[158]. Fibroscan® value was significantly reduced following successful bilirubin drainage. Likewise, vascular hepatic congestion can erroneously increase Fibroscan® values. This effect is entirely reversible upon correction of cardiovascular dysfunction[150]. Fasting is also important to avoid overestimation of Fibroscan® measurement. A study by Arena *et al*[159] showed the confounding effect of a meal on the accuracy of liver stiffness in CHC patients. The authors proposed a fasting period of 120 min before performing the examination. On the same line, Berzigotti *et al*[160] demonstrated that post-prandial hyperemia is accompanied by a marked increase in liver stiffness in patients with liver cirrhosis.

Transient elastography by using Fibroscan® is a highly reproducible technique[161]. Inter- and intra-observer fluctuations are affected by high grade hepatic steatosis, mild fibrosis (F1-F2 by METAVIR) and a BMI $\geqslant$ 25 kg/m^2[161]. Nevertheless, the applicability of Fibroscan® may not be as good as that of biomarkers. Overall, in a study of 13369 examinations, liver stiffness data were not interpretable in nearly 20% of cases, mainly due to failure to obtain reliable measurements according to the manufacturer's recommendations. The technical limitations were attributed to obesity of patients, and in particular to increased waist circumference, and to limited experience of the operator[162].

COMBINATION ALGORITHMS OF NON-INVASIVE METHODS FOR ASSESSMENT OF LIVER FIBROSIS

In order to increase the diagnostic performance of the single method, especially for the diagnosis of significant fibrosis, non-invasive methods have been combined in diagnostic algorithms. The rationale is to combine non-invasive methods, such as Fibroscan® and serum biomarkers, or different, unrelated serum biomarkers. Such a strategy led to a significant reduction in the number of liver biopsies and to an increase in diagnostic accuracy, and it has been recommended by guidelines, such as those from the EASL and CASL. In a recent review Pinzani *et al*[163] suggested to apply two unrelated non-invasive methods in CHC patients, and to obtain liver biopsy in only one subgroup of them. On the same line, Manning and Afdhal[164] have proposed to perform annually biomarkers analysis *plus* Fibroscan®. The utilization of combination algorithms does not completely eliminate the need for liver biopsies; however it can greatly reduce it and limit it to cases where serum biomarker data do not show a reliable accuracy. Combination algorithms used in clinical settings are able to provide the subsequent responses: (1) Presence or absence of significant liver fibrosis, which indicates whether to administer antiviral therapy or not; (2) Presence or absence of liver cirrhosis, which indicates whether to proceed with specific screening for esophageal varices and HCC or not; and (3) Liver biopsy needed to correctly stage hepatic fibrosis. Combination algorithms of non-invasive methods for assessment of liver fibrosis that have been proposed in the literature are summarized in Table 7.

STEPWISE COMBINATION ALGORITHMS

Sebastiani *et al*[133,165] proposed an approach that combines APRI and Fibrotest® sequentially. These methods were selected because they are highly validated and widely available. The Sequential Algorithm for Fibrosis Evaluation (SAFE) biopsy was aimed at reducing the amount of liver biopsies needed to accurately stage liver fibrosis, and at minimizing misclassifications. The stepwise modeling of

Table 7 Combination algorithms of non-invasive methods for liver fibrosis proposed in chronic hepatitis C

Algorithm's name	Type	Non-invasive methods adopted	AUC for ⩾ F2	AUC for F4	Saved liver biopsies for > F2 (%)	Saved liver biopsies for F4 (%)	Number of studies (patients)
SAFE biopsy[133,165]	Stepwise	APRI, Fibrotest®	0.89-0.94	0.87-0.92	43.8-54.0	74.8-93.4	6 (4118)
Bordeaux algorithm[167,168]	Synchronous	Fibrotest, Fibroscan®	0.88-0.91	0.93-0.95	71.9-77.0	78.8-79.0	3 (875)
Leroy algorithm[124]	Synchronous	APRI, Fibrotest®	0.94	NA	19.0-29.2	NA	3 (1381)
Fibropaca algorithm[134]	Synchronous	APRI, Fibrotest, Forns' index	0.88	0.85	51.7	76.2-81.3	2 (1248)
Angers algorithms[171]	Synchronous	Fibrotest, Fibrometer®	0.892	0.917	79.8	89.7	1 (390)
Bourliere's algorithm[166]	Stepwise	APRI, Hepascore	91%-96% (accuracy)		33-45		1 (467)
Fibrometer® + Fibroscan[172]	Synchronous	Fibrometer, Fibroscan	86.7%		100		1 (1785)

APRI: Aspartate aminotransferase to platelet ratio index; NA: Not available.

the algorithms for significant liver cirrhosis and fibrosis was intended for achieving ⩾ 90% accuracy. The model uses APRI as a first line test because of its simplicity and low cost, and Fibrotest® as a second line test because of its accuracy and higher cost. Importantly, it uses liver biopsy as a third line test only in cases where the combined non-invasive biomarkers fail to classify with adequate accuracy. The modeling of the stepwise algorithms was established on the single biomarkers predicted values. The SAFE biopsy has been validated by data obtained in a multi-centered study with more than 2035 CHC patients (Table 7). They show excellent diagnostic performance and substantial reduction of liver biopsies (50% for significant fibrosis and 80% for cirrhosis). Another proposed stepwise algorithm combines Hepascore®, a patented test, with APRI[166]. This approach yielded 91% diagnostic accuracy and reduced liver biopsies for significant fibrosis by 45%. To date, its main drawback is the lack of extensive validation data for Hepascore®, as compared to APRI, Fibrotest® and Forns' index.

SYNCHRONOUS COMBINATION ALGORITHMS

Castéra *et al*[167] proposed the Bordeaux algorithm, which combines Fibrotest® and Fibroscan®. This approach improves accuracy for the diagnosis of significant fibrosis. Performance of the Bordeaux algorithm and SAFE biopsy was subsequently compared in 302 patients with CHC[168]. Both algorithms saved a high number of liver biopsies to diagnose cirrhosis, while the Bordeaux algorithm was more effective in the prevention of liver biopsies for the diagnosis of significant fibrosis. The accuracy of the two algorithms was similar for significant fibrosis, whereas, the Bordeaux algorithm was more accurate for the diagnosis of cirrhosis. Nevertheless, the Bordeaux algorithm requires the use of Fibrotest® and Fibroscan® in all patients, which increases cost. The SAFE biopsy is much cheaper because it requires the use of Fibrotest® only in a subgroup of patients who cannot be categorized by APRI.

Another combination algorithm consisting of Forns' index, Fibrotest® and APRI was proposed by Bourliere *et al*[169] and showed an exceedingly good performance for diagnosing both significant fibrosis and cirrhosis, saving around 50% and 80% of liver biopsies, respectively. Leroy *et al*[124], proposed a synchronous algorithm using Fibrotest® and APRI in concordance, which demonstrated exceptional performance in the diagnosing of significant fibrosis. However, the number of saved liver biopsies was relatively small as compared to the other combination algorithms.

The SAFE biopsy, Fibropaca algorithm and Leroy algorithm were applied to 1013 CHC patients[170]. The accuracy of the Fibropaca algorithm and the SAFE biopsy was similar; however, the SAFE biopsy reduced the number of biopsies and required the acquisition of fewer non-invasive biomarkers, thereby saving costs. Boursier *et al*[171] described the Angers' algorithm, which combines Fibrotest® and Fibrometer®, and showed that this could save 44.8% of liver biopsies by exhibiting an overall accuracy of 95.3%. Moreover, they suggested that the synchronous combination algorithms could be more efficient than the sequential algorithms, including SAFE biopsy, which is at present debatable. On the same line, a study of 1785 CHC patients compared the performance of eight diagnostic algorithms[172]. The authors found an impressive 0% rate in liver biopsy need with a synchronous combination of Fibroscan® and Fibrometer®. However, even though it showed an excellent accuracy, Fibrometer® has been less evaluated independently compared to other established tests that are used for SAFE biopsy and Bordeaux algorithm (APRI, Fibroscan® and Fibrotest®), and is not licensed in as many countries as Fibrotest®.

In conclusion, combination algorithms can significantly improve the diagnostic accuracy of the single non-invasive method, particularly to diagnose significant liver fibrosis. Moreover, they can safely reduce the number of liver biopsies needed in clinical practice. The choice of the algorithm to be used in clinical practice may be based on some considerations: (1) what is locally available; (2) what is more validated; (3) what is not affected by patient co-morbidities; and (4) which methods the physicians feel more comfortable with.

MONITORING OF COMPLICATIONS IN LIVER DISEASE

Several studies suggest that complications of liver dis-

ease in compensated cirrhosis can be monitored by non-invasive techniques. As such, values of liver stiffness in cirrhotic patients increase with the progression of liver disease. In a retrospective study of 711 patients, values of liver stiffness significantly correlated with the severity of chronic liver disease in terms of Child-Pugh score, clinical parameters (ascites, varices, history of bleeding, HCC), biochemical parameters (albumin, bilirubin, platelets and INR) and other indications (large esophageal varices, splenomegaly on sonography, nodular surface, heterogeneous parenchyma)[152]. Fibroscan® cut-off values of 27.5, 49.1, 53.7 and 62.7 kPa had > 90% NPV for large esophageal varices, history of ascites, HCC and esophageal bleeding, respectively. On the same line, Vizzutti *et al*[173] reported a correlation between liver stiffness and portal hypertension, as assessed by the hepatic venous pressure gradient (HVPG). A cut-off of 17.6 kPa of Fibroscan® had 90% sensitivity to rule-in esophageal varices.

In a study of 99 cases Fibrotest® showed a high NPV (100%) to exclude large esophageal varices with a cut-off value of 0.75 in detecting large varices[174]. In another study of 70 patients, Fibrotest® showed 92% NPV for excluding large esophageal varices with a specific cut-off (0.78), with an overall AUC of 0.75; Fibroscan® showed an AUC of 0.87[137]. A low platelet count has been related to the presence of esophageal varices. The discriminating threshold ranged between 68000 and 160000/mm^3[137,175]. However, other studies concluded that platelet count is not an adequate non-invasive marker for esophageal varices[176]. For the diagnosis of esophageal varices, Giannini *et al*[177] reported an overall accuracy of 86% and good sensitivity at 91.5%, and with the cut-off platelet count to spleen diameter ratio at 909.

The value of 7 non-invasive biomarkers of liver fibrosis in prediction of esophageal varices was investigated in one study with 510 patients with cirrhosis[175]. The presence of esophageal varices could be excluded with ⩾ 96% NPV by Lok index with the cut-off of 1.5. Importantly, a combination of Forns' index (8.5 cut-off) and Lok index (0.9 cut-off) could rule-out clinically significant esophageal varices, defined as varices requiring primary prophylaxis of bleeding (large esophageal varices or small varices with red signs or in Child-Pugh class C), with 91% NPV. Likewise, a good performance of Lok index for diagnosis of varices was also reported by Castéra *et al*[137], with a 0.87 AUC.

Complications of liver cirrhosis, including esophageal varices, ascites and hepatic encephalopathy, occur when portal hypertension develops. The gold standard of reference to diagnose portal hypertension, measurement of HVPG, is invasive and limited to highly specialized centers. Berzigotti *et al*[178] demonstrated that liver stiffness measurement by Fibroscan® predicts presence of portal hypertension with an AUC of 0.88 as compared to HVPG. Moreover, the performance increased significantly when Fibroscan® was combined with platelets or spleen size (up to 0.935 AUC). In a study of 100 consecutive patients with CHC, spleen stiffness was demonstrated to predict accurately HVPG. Moreover, a cut-off value of spleen stiffness of 41.3 was able to rule-out esophageal varices with 98% sensitivity and 66% specificity[179].

Even though at present non-invasive methods for liver fibrosis cannot replace endoscopy for screening of esophageal varices, they may help stratifying cirrhotic patients for risk classes and possibly reducing the number of endoscopies.

PROGNOSTIC VALUE OF NON-INVASIVE METHODS FOR LIVER FIBROSIS

Evaluating the stage of liver fibrosis is a key point not only for management of the patient, but also for long-term prognosis. If CHC patients have mild fibrosis at diagnosis, only 25%-30% of them progress to become cirrhotic within 20 years. However, virtually all patients diagnosed with portal fibrosis will progress to liver cirrhosis within 18-20 years, whereas all patients diagnosed with septal fibrosis will progress to cirrhosis in only 8-10 years. Moreover, end-stage complications mainly occur in patients with advanced disease. Portal hypertension, ascites, or HCC are associated with a shorter survival. Given that the level of fibrosis predicts liver-related complications and survival, early assessment of the risk of bad prognosis helps the physician to manage patients with cirrhosis and to make decisions about liver transplantation.

Liver biopsy does not meet the criteria for serial monitoring and surrogate end-point marker tool because of its invasiveness, sampling error, intra- and inter-observer variability, cost, and patient reluctance to undergo serial monitoring. As such, the value of non-invasive methods for liver fibrosis in predicting clinical outcomes of CHC has been investigated. Ngo *et al*[180] showed that Fibrotest-Fibrosure® displays a significant correlation with survival, with a 5-year prognostic value similar to that of liver biopsy for the prediction of cirrhosis decompensation and survival. Along the same line, Nunes *et al*[181] showed that hyaluronic acid, APRI, and Fib-4 were significantly associated with mortality. An association between liver stiffness and risk of HCC development in CHC patients was also described[182].

A definitive demonstration of the long-term predictive role of non-invasive methods for liver fibrosis comes from a study by Vergniol *et al*[183]. In a consecutive cohort of 1457 CHC patients, the researchers investigated the role of Fibrotest-Fibrosure®, APRI, Fib-4 and liver stiffness in predicting death, liver-related death, and liver transplantation during a 5-year follow-up period[183]. All non-invasive fibrosis methods could predict shorter survival, with liver stiffness and Fibrotest® showing the higher predictive values. Moreover, patient outcomes worsened as liver stiffness and Fibrotest® values increased. On the same line, a recent study of 3927 patients with CHC showed that Fibrotest® and Fibroscan® predicted 10 years occurrence of severe liver-related complications, HCC,

variceal bleeding and hepatic failure[184].

A study recently performed in our center investigated the value of Fibroscan® in diagnosing subclinical cirrhosis, as defined by liver stiffness ⩾ 13 kPa and absence of thrombocytopenia, ultrasonographic signs of advanced liver disease/splenomegaly, esophageal varices, and ascites[185]. In 1492 consecutive patients with a mean follow-up of 18 mo, we found that patients with subclinical cirrhosis had a higher incidence of cirrhosis-related events as compared to non-cirrhotic patients, including HCC. We then concluded that screening with Fibroscan® may help early identification of subclinical cirrhosis, stratifying patients by risk and establishing a surveillance program for HCC and varices.

NON-INVASIVE METHODS FOR LIVER FIBROSIS AND ANTIVIRAL TREATMENT: MONITORING, RESPONDING, REGRESSING

Antiviral therapies for CHC are medium term and expensive, and it may be clinically worthy to monitor histological data, in addition to virological and biochemical responses. Even in the rapidly changing panorama of antiviral therapy against HCV infection, the cost will remain a major issue. Initial data revealed significant alterations of Fibroscan® and Fibrotest® values in CHC patients during and after antiviral therapy. In 91 patients with CHC, Hezode *et al*[186] investigated the kinetics of liver stiffness during antiviral treatment with pegylated interferon alpha and ribavirin. A significant improvement in liver stiffness was observed during therapy, which continued after treatment only in patients who achieved SVR. Interestingly, similar dynamics of liver stiffness were observed in cirrhotic *vs* non-cirrhotic patients. In multivariate analysis, only the SVR was associated with long-term improvement of liver stiffness. The authors hypothesized that these changes reflect fibrosis regression. This is in keeping with reported improvement of histology in pair liver biopsies[187,188]. On the same line, patients were more likely to achieve SVR if the baseline value of Fibroscan® or Fibrotest® was lower, and mean value of patients at end of treatment was lower in responders[189]. Taken together, these data suggest that antiviral therapies promote regression of liver fibrosis. Larger prospective studies are required for further validation.

CONCLUSION

Staging of liver fibrosis is crucial for the management of CHC patients and for prognosis. Liver biopsy cannot be used as a screening tool due to its invasiveness and drawbacks, especially in light of recent recommendations for large scale screening against HCV infection. Non-invasive methods to stage liver fibrosis are accurate, cost-effective and patient-friendly. Combination algorithms can help optimize the implementation of non-invasive methods in clinical practice. A rational approach is to perform a first line screening of liver fibrosis with algorithms combining the most accredited non-invasive methods and to perform a biopsy only for patients where non-invasive tests yielded unreliable or inaccurate results. Non-invasive methods for assessment of liver fibrosis can also predict cirrhosis-related complications and long-term outcomes of CHC patients. Thus, they can be used to stratify patients by risk classes and to prioritize for antiviral treatment and liver transplantation. Finally, non-invasive methods can be used to monitor the regression of liver fibrosis in response to antiviral therapy.

ACKNOWLEDGMENTS

We thank Lynda Lennox for editing the manuscript.

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P- Reviewer: Adler MG, Fraquelli M, Yoshioka K
S- Editor: Ma YJ **L- Editor**: A **E- Editor**: Ma S

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DOI: 10.3748/wjg.v20.i32.11054

World J Gastroenterol 2014 August 28; 20(32): 11054-11061
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (2): Hepatitis C virus

Hepatitis C virus and diffuse large B-cell lymphoma: Pathogenesis, behavior and treatment

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Supported by In part by grants of AViLL/AIL (Associazione Vicentina per le Leucemie, i Linfomi e il Mieloma/Associazione Italiana Leucemie) (Vicenza, Italy); the Hematology Project Foundation (HPF, Fondazione Progetto Ematologia, Vicenza, Italy)
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Received: December 24, 2013 Revised: February 25, 2014
Accepted: May 29, 2014
Published online: August 28, 2014

Abstract

A significant association between hepatitis C virus (HCV) infection and B-cell lymphoma has been reported by epidemiological studies, most of them describing a strong relationship between indolent lymphomas and HCV. Furthermore, the curative potential of antiviral therapy on HCV related indolent lymphomas supports a specific role for the virus in lymphomagenesis. These observations are reinforced by numerous laboratory experiments that led to several hypothetical models of B-cell transformation by HCV. Diffuse large B-cell lymphoma (DLBCL), the most common lymphoma subtype in the western countries, has been associated to HCV infection despite its aggressive nature. This association seems particularly prominent in some geographical areas. Clinical presentation of HCV-associated DLBCL has consistently been reported to differ from the HCV-negative counterpart. Nevertheless, histopathology, tolerance to standard-of-care chemo-immunotherapy (R-CHOP or CHOP-like regimens) and final outcome of HCV-positive DLBCL patients is still matter of debate. Addition of rituximab has been described to enhance viral replication but the probability of severe hepatic complications remains low, with some exceptions (*i.e.*, hepatitis B virus or immune immunodeficiency virus co-infected patients, presence of grade > 2 transaminases elevation, cirrhosis or hepatocarcinoma). HCV viral load in this setting is not necessarily directly associated with liver damage. Overall, treatment of HCV associated DLBCL should be performed in an interdisciplinary approach with hepatologists and hematologists with close monitoring of liver function. Available reports reveal that the final outcome of HCV-positive DLBCL that receive standard immunochemotherapy is not inferior to their HCV-negative counterpart. This review summarizes data on epidemiology, pathogenesis and therapeutic approach on HCV-associated DLBCL. Several issues that are matter of debate like clinical management of patients with transaminase elevation, criteria for discontinuing or starting immuno-chemotherapy, as well as the exact role of monoclonal antibodies will be analyzed.

Key words: Hepatitis C virus; Non-Hodgkin lymphoma; Liver; Toxicity; Diffuse large B-cell lymphoma; Rituximab; Cyclophosphamide; Hydroxydaunorubicin; Vincristine; Prednisolone; Immuno-chemotherapy; Antiviral treatment

Core tip: Patients with hepatitis C virus-positive diffuse large B-cell lymphoma should be managed in a multidisciplinary setting. Initial evaluation of liver status and comorbidities is essential to establish if the patient is candidate to curative approaches. Unless contraindicated by adverse clinical conditions, patients should be treated with standard immuno-chemotherapy. Concomitant hepatitis B virus infection and liver failure or cirrhosis confer a significantly higher risk of viral reactivation or therapy related complications. These patients

should be managed cautiously and treated with less intense approaches at least for the initial cycles. Antiviral treatment should be considered after the end of immuno-chemotherapy, when lymphoma remission has been achieved.

Visco C, Finotto S. Hepatitis C virus and diffuse large B-cell lymphoma: Pathogenesis, behavior and treatment. *World J Gastroenterol* 2014; 20(32): 11054-11061 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11054.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11054

INTRODUCTION

Several reports from countries with relatively high prevalence of hepatitis C virus (HCV) infection have documented a significant epidemiological association between HCV infection and development of B-cell non Hodgkin's lymphoma[1-4]. A direct role of the virus in lymphomagenesis is primarily suggested by the prominent curative potential of antiviral therapy on HCV-related B-cell proliferation or low-grade B-cell lymphomas[5-8]. Such an effect implies a specific role for the virus in maintaining B-cell proliferation, although the exact mechanism remains unknown[9,10].

Diffuse large B-cell lymphoma (DLBCL) is the most common lymphoma subtype in the western countries and is characterized by aggressive clinical behavior. Despite its pathological heterogeneity[11], it is commonly managed with the combination of chimeric monoclonal antibody against the protein CD20 (rituximab) and polichemotherapy (immuno-chemotherapy) in the front-line setting. The available literature regarding the pathobiology and management of patients with DLBCL in the setting of HCV is quite limited compared to low-grade B-cell lymphomas. Indeed, several recent retrospective studies[12-18] have reported that patients with HCV related DLBCL have peculiar characteristics compared to their HCV-negative counterparts, suggesting a possible influence of the virus since the very early steps of lymphomagenesis. HCV-positive patients are usually older, have more frequent spleen/liver or extranodal involvement and elevated lactate dehydrogenase. However, epidemiological data are among the strongest argument in favor of a role of the viral infection in the development of DLBCL. Due to the lack of prospective studies, tolerance to chemo-immunotherapy and outcome of HCV-positive patients with DLBCL are controversial. Several issues like clinical management of patients with transaminases elevation, criteria for discontinuing or starting immuno-chemotherapy in the event of escalation in HCV replication in an asymptomatic patient, as well as the exact role of monoclonal antibodies, remain unclear.

EPIDEMIOLOGY

Over the past two decades considerable evidence has accumulated on the association between HCV and hematologic malignancies, most notably B-cell pre-malignant and malignant proliferations. Early results[19] reported a strong association of HCV with lymphoplasmacytic lymphoma/Waldenstrom macroglobulinemia that were possibly related to the association existing between HCV-infected patients and type Ⅱ mixed cryoglobulinemia[20], which may sometimes represent a pre-malignant disorder. Later on, a strong association emerged also in regard to marginal zone lymphomas and DLBCL[21]. Epidemiological works demonstrated that the relative risk of lymphoma was significantly higher in geographical areas with high rather than with low HCV prevalence. In fact, several studies[22-24] from countries with low HCV prevalence did not observe such association, although relying on small numbers of patients. A recent larger report from Sweden[25], where the HCV prevalence among healthy population is low, reported an increased risk of lymphoma occurrence among HCV infected patients also in this northern country. Another large-base survey on 4784 adult cases of non-Hodgkin's lymphoma and 6269 age- and sex-matched controls from an International Lymphoma Epidemiology Consortium including centers from Spain, North America and Australia[21]reported a significantly increased risk in HCV infected patients for development of marginal zone lymphoma (OR = 2.47; 95%CI: 1.44-4.23), DLBCL (OR = 2.24; 95%CI: 1.68-2.99), and lymphoplasmacytic lymphoma (OR = 2.57; 95%CI: 1.14-5.79). An Italian case-control study[2] reported an even higher association of HCV infection with DLBCL (OR = 3.5) with respect to indolent/low-grade B-cell lymphomas (OR = 2.3), suggesting that approximately 1 out of 20 cases of incidental DLBCL in Italy may be attributable to HCV. Interestingly, development of DLBCL in these patients was not reported to be preceded by cryoglobulinemia or indolent lymphomas, which pointed to the association of HCV with *de-novo* DLBCL not transformed from a previous low-grade entity.

Overall, partly due to the worldwide distribution of HCV infection, the association of HCV infection with lymphomas appears more relevant in Southern Europe, Turkey, Egypt, other Mediterranean countries[26], Taiwan[27-30] and Japan[4,31]. Its role in Northern America[21] seems to be emergent, while other countries like England and Scotland[32], India[33], France[22] or Thailand[23] have reported much lower incidence and impact of this association in the clinical practice. Large scale studies including higher numbers of patients are warranted from regions with low HCV prevalence before driving conclusions on the association between virus and lymphomas in these areas.

PATHOGENESIS

Some important arguments in the literature describe a possible pathogenetic role of HCV infection in the development of aggressive B-cell lymphomas. Cumulative evidence have suggested an HCV-related antigen driven process in lymphoma development, similarly to what ob-

served with *Helicobacter pylori* and lymphoid proliferation in mucosa-associated lymphoid tissue. However, even if recent progress in better understanding HCV-related lymphoproliferations has been made, the precise relationship between HCV and lymphoma development remains to be clarified.

Some recent observations are in favor of an active role of the virus in lymphomagenesis. HCV transgenic mice expressing the full HCV genome in B-cells have been shown to develop DLBCL in 25% of cases[34]. Moreover, HCV has been shown to protect human B lymphocytes from Fas-mediated apoptosis *via* E2-CD81 engagement, even in the absence of viral entrance into the human B-cell[35]. Finally, the viral core and NS3 proteins were responsible for the inhibition of DNA repair, mediated by nitric oxide and reactive oxygen species in another study[36]. Stable expression of core protein induced frequent chromosome translocations in cultured cells and in transgenic mice, describing an HCV mediated inhibition of DNA damage-repair and enhancement of chromosomal breaks[36]. It must be noted that B-cell associated virus can readily infect hepatoma cells, harboring an enhanced infectivity compared to extracellular virus. According to this theory, the virus can modify the normal tropism of B-cells, escape natural immunity and survive in the infected liver[37].

Based on these and others experiments, several hypothetical models of B-cell transformation by HCV have been formulated[38,39]. A direct transformation model, where HCV would directly infect B cells, possibly through CD81-E2 interaction, expressing its oncogenic potential through cellular nitric oxide-synthase and NS3/4 mediated mutations of proliferation genes. On the other, an indirect transformation model would rely on the interaction between E2 and CD81 on the cell surface, which would induce expression of activation-induced deaminase and somatic hypermutation of immunoglobulin genes and potential proto-oncogenes, inducing a sustained B cell stimulation. Finally, the so called "hit and run" theory, which relies on virus-induced genetic damage of B-cells caused by a transiently intracellular virus [*e.g.*, mutation of tumor suppressor genes (p53, BCL-6, beta-catenin)]. All theories may imply a role for microRNA dysregulation, since recently a key role of miR-26b downregulation has been suggested in undermining tumor suppression[40,41].

In addition to its being hepatotrophic, HCV is also a lymphotrophic virus that is able to infect and replicate within peripheral blood mononuclear cells, as witnessed by its association with mixed cryoglobulinemia type Ⅱ, which is characterized by clonal expansion of B cells. An increased expression of the BCL2 oncogene, mediated by a high prevalence of t(14;18) translocation has been detected in peripheral blood mononuclear cells of HCV infected patients by polymerase chain reaction[42]. Furthermore, the disappearance of the t(14;18) translocation following antiviral treatment was strongly associated with virologic response[5]. Altogether, these findings again suggest a possible pathogenetic link between HCV and aggressive lymphomas, which are known to be characterized by BCL2 translocation in around 20% of *de-novo* DLBCL[43].

Lymphomas that develop in HCV-infected patients seem to combine disease-specific signatures and different sets of genes whose expression is associated with B-cell receptor activation and specific nuclear factor kappa-light-chain-enhancer of activated B cells transcription factors, including BCL2 translocations[43]. Identification of molecular signatures in lymphomas occurring in the HCV-infected population could facilitate a more rational approach to the diagnosis as well as more tailored treatments, also in DLBCL[44].

CLINICAL MANAGEMENT AND TOLERANCE TO TREATMENT

In the management of HCV-associated DLBCL, anthracycline-based chemotherapy [usually cyclophosphamide, hydroxydaunorubicin, vincristine, prednisolone (CHOP)] associated with rituximab (immuno-chemotherapy) is the standard of care[45]. Differently from indolent B-cell lymphomas, antiviral treatment yet does not play a significant role in HCV-positive DLBCL.

Since the treatment of DLBCL is usually based on rapidly active drugs due to the aggressive clinical behavior of the disease, antiviral regimens have not been tested so far on large series, especially due to their slow effect on lymphoid proliferation, if any. Nevertheless, few anecdotal reports that show successful antiviral treatment in patients with DLBCL[46,47] or mantle cell lymphoma[48] suggest that these lymphomas might also be sensitive to a drop in the viral load, as is the case of low-grade lymphomas. Sequential immune-chemotherapy followed by antiviral therapy has been claimed in two preliminary reports with promising results[49,50], but more mature and prospective data are eagerly awaited.

Due to the lack of prospective studies, tolerance to chemo-immunotherapy and outcome of HCV-positive patients with DLBCL are controversial. Moreover, the literature existing on HCV-positive DLBCL is unbalanced on the side of hematologists, with scanty detailed reports on the hepatic side. It has been reported that the addition of rituximab in the R-CHOP regimen may add hepatotoxicity, favor HCV reactivation or acceleration of viral liver inflammation[16,51]. In a benign disorder like HCV-associated mixed cryoglobulinemia, rituximab therapy have demonstrated an excellent safety and tolerability profile, including lack of viral or hepatic flares[52,53]. Patients with aggressive B-cell lymphoma that undergo anthracycline-based chemotherapy coupled with rituximab (R-CHOP) usually have good tolerance to the combination of drugs[54], but a less beneficial safety profile than rituximab monotherapy in benign diseases. Most frequent complications are represented by hepatic flares. Immunochemotherapy can enhance viral replication, especially in the presence of hepatitis B virus (HBV) or human immunodeficiency virus co-infections or when older or particularly immunocompromised

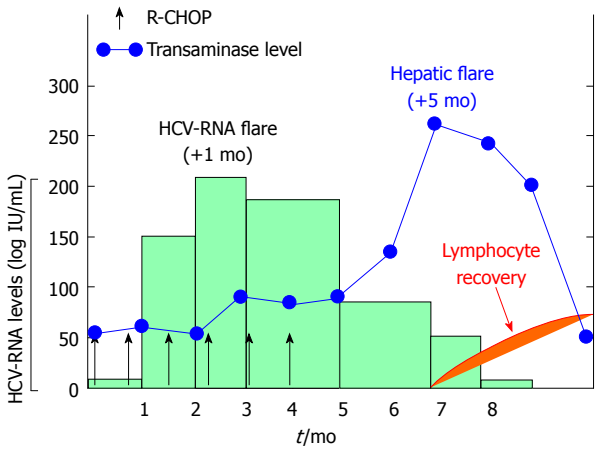

Figure 1 Speculative illustration. Speculative illustration of expected hepatitis C virus (HCV)-RNA load and transaminase flare during and after rituximab cyclophosphamide, hydroxydaunorubicin, vincristine, prednisolone (R-CHOP) immmunochemotherapy for a diffuse large B-cell lymphoma ideal patient (not cirrhotic, no other risk factors) experiencing transient toxicity. Curves and histograms reflect the average behavior of patients reported so far in the literature.

patients are treated[55]. Indeed, hepatic complications of grade 3 or more (according to the Common Terminology Criteria for Adverse Events v4.0) are quite unusual, but symptomatic liver dysfunction, elevation of aspartate aminotransferase/alanine aminotransferase levels, fibrosis, decompensated cirrhosis, and reactivation of chronic hepatitis may occur[31]. One study reported poor tolerance to intensive chemotherapy including autologous stem cell transplant[16], but most studies have described acceptable liver toxicity[14,15,17,18].

Whether rituximab causes an additive negative effect on hepatotoxicity is still a matter of debate as only few systematic comparative data exist[56]. After CHOP without rituximab hepatitis flares have been described in some cases, occurring 2 to 3 wk after the end of the cycle, grade 3-4 liver toxicity was in the range of 10% to 28%, and fatal complications were rare. It must be noted that prednisone therapy alone can increase HCV-RNA levels in chronic hepatitis patients (but decrease transaminase levels). With the addition of rituximab, hepatitis flares have been described in 26% to 33%, being significantly more frequent than in control series of patients with HCV-negative DLBCL (2%-3%)[14,56-60]. In all studies major toxicities were rare. Treatment discontinuation due to liver function impairment has been described in 0% to 11% of patients, while death due to liver failure in 0% to 5%[57,59,60]. Severe hepatotoxicity was observed in 14% of patients in a recent large Italian multicenter survey[59]. The use of rituximab was not associated with increased rate of hepatotoxicity (P = 0.5) compared to patients receiving chemotherapy only or localized approaches. Furthermore, severe hepatic toxicity was not associated with poor progression-free survival or overall survival in patients who were HCV-positive[60].

Clinical and serological behaviour of HCV viral load and transaminase level after an ideal immunochemotherapy with 6 courses of R-CHOP, based on available literature, in a patient without known risk factors, is summarized in Figure 1. Median time from start of treatment to development of severe hepatic failure is expected around the 100th day, but the range is wide, with cases reported since the first 10 d after R-CHOP up to 320 d after. Importantly, no relation was described between pre-treatment HCV-RNA levels and hepatic toxicity afterwards, with two patients that developed severe hepatic toxicity but had low HCV-RNA levels before treatment. This finding was substantiated by other studies[57,59,60]. However, patients presenting with cirrhosis or compromised liver function usually experience earlier toxicity. HCV-RNA levels dramatically falls at the time of increase of the transaminases, suggesting that the cause of liver damage is an immune reaction against hepatocytes, again supporting the notion that HCV viral load is not necessarily directly associated with liver damage[57]. B-cell recovery is also implied, with progressive restoration of immune homeostasis that seems to contribute to liver function recovery[61].

Despite the lack of productive infection (no replication of the virus inside B-cells), it has been demonstrated that B cells lysed by effector blood cells could release up to four times more infectious virus following rituximab treatment. These data support a role for rituximab lysis of B cells and release of infectious HCV[62]. Hepatitis C viremia was shown to increase to approximately twice the baseline level in the responders after rituximab monotherapy, whereas it remained much the same in the non-responders[63]. Viral genotype might also condition chemotherapy induced hepatotoxicity[51,64].

A recent report described two patients with DLBCL and HCV-related liver cirrhosis experiencing grade > 3 increase in transaminases and ascites while being treated with R-CHOP. Treatment with ribavirin 1000 mg/d was started. Both patients had a rapid decrease in alanine aminotransferase levels and disappearance of the ascitic fluid, suggesting a possible benefit of treating HCV infection when patients experience hepatic flare, also in the course of chemotherapy[65].

In conclusion, we suggest to monitor transaminase levels at least twice during the induction cycle, and then once every first day of each R-CHOP cycle in patients with standard risk HCV-positive DLBCL. RNA viral load should be measured at screening and then re-evaluated only in case of transaminase alterations, to rule out different causes of hepatic toxicity. When sudden transaminase increase occurs, subsequent cycles should be postponed before deciding to definitively stop treatment. More frequent controls of transaminase levels and eventually of RNA levels should be reserved to patients with baseline elevated transaminases or with underlying hepatic complications of HCV infection (including cirrhosis, concomitant HBV infection and hepatocarcinoma)[51,57,66,67].

Table 1 Clinical outcome of hepatitis C virus-positive diffuse large B-cell lymphoma patients according to retrospective studies

Ref.	DLBCL-HCV positive patients (*n*)	Treatment	PFS/EFS (3 yr)	OS (3 yr)
Besson *et al*[16]	26	Intensive protocols	53%	56%
Visco *et al*[14]	156	CHOP and CHOP-like/R-CHOP	60%	80%
Park *et al*[15]	32	CHOP(18)/R-CHOP(11)	54.7% at 5 yr	59.2% at 5 yr
Tomita *et al*[17]	25	CHOP/CHOP-like	NA	46%
Ennishi *et al*[60]	131	R-CHOP	69%	75%
Merli *et al*[59]	535	CHOP(214)/+R-CHOP (252)	53% (58% for R-CHOP)	68% (71% for R-CHOP)

DLBCL: Diffuse large B-cell lymphoma; CHOP: Cyclophosphamide, hydroxydaunorubicin, vincristine, prednisolone; PFS/EFS: Progression-free survival/event-free survival; OS: Overall survival; HCV: Hepatitis C virus.

CLINICAL PRESENTATION AND OUTCOME

In some recent retrospective studies[12-18], patients with HCV related DLBCL have been described to be usually older, to have more frequent extranodal involvement (especially spleen and liver) and elevated lactate dehydrogenase (LDH) compared to their HCV-negative counterparts. Results regarding the prevalence of transformed DLBCL as compared with *de-novo*-DLBCL are contradictory. These results suffer of the retrospective selection of patients as well as of the absence of pathology review in largest published series[14,16,59,60].

Primary hepatic and primary splenic DLBCL are rare entities that have been reported to display an association with HCV infection, with outcomes that were quite favorable[18]. Persistent human hepatitis virus infections, especially HCV, have been also claimed to play an important role in the tumorigenesis of splenic DLBCL in Japan[68].

The outcome of HCV-positive DLBCL has been shown to be not inferior to that of their HCV-negative counterpart once patients are adequately treated. However, this issue is still a matter of debate. As previously mentioned, HCV-positive DLBCL patients display specific presentation potentially affecting clinical features included in prognostic scores (*i.e.*, age, number of extranodal sites, stage). Furthermore, LDH, which is a mainstay of the international prognostic index (IPI), is potentially biased in HCV-positive patients, as it is influenced not only by lymphoma but also by chronic HCV infection. Notably, the IPI was built and validated in series comprising only HCV-negative patients or with unknown HCV-status, while their predictive significance has never been formally validated in HCV-positive DLBCL[59]. The Fondazione Italiana Linfomi has recently carried out a large multicenter retrospective study with the aim of constructing a new prognostic system for HCV-associated DLBCL describing an "HCV Prognostic Score" based on performance status, albumin level and HCV-RNA viral load, which appeared extremely reliable[59]. Concomitant HBV infection or HBV related hepatocarcinoma has been reported to confer an extremely negative influence on outcome of these patients[14,60].

Few studies evaluated the clinical outcome of HCV-positive DLBCL (Table 1). In the pre-rituximab era, Besson *et al*[16] reported a worse overall survival (OS) in HCV-positive DLBCL patients treated with intense chemotherapeutic protocols. On the contrary, the study by Ennishi *et al*[60] on 131 HCV-positive patients treated with R-CHOP reported a similar outcome in HCV-positive patients compared to HCV-negative (3-year OS 75% *vs* 84%, $P = 0.07$). The outcome of patients treated with R-CHOP in the study by Merli *et al*[59] (3-year OS 71%) was similar to the latter study reflecting expected figures for a similarly treated DLBCL population of similar age with no HCV infection (*i.e.*, RICOVER-60 study, 3-year OS 72%). Neither HCV infection itself nor the development of severe hepatic toxicity was correlated with survival, suggesting that HCV positive patients derive a similar antilymphoma benefit from the addition of rituximab.

CONCLUSION

Patients with HCV-positive DLBCL should be managed involving different specialists, with the hematologist and the hepatologist both involved from the beginning in the treatment program. Initial evaluation of the liver status and of comorbidities is essential to establish if the patient is candidate to curative approaches including chemotherapy and monoclonal antibodies. Liver biopsy is not mandatory in all cases in our opinion, but its indication should be carefully evaluated with the hepatologist in case a cirrhosis is suspected. Presentations with concomitant HBV infection or liver cirrhosis represent two common clinical issues. Such patients need to be informed before starting chemotherapy that they will have a significantly higher risk of viral reactivation, and serial controls of their liver function are mandatory since the very first weeks of cytotoxic treatment. Unless contraindicated by the hepatologist due to older age or particular comorbidities, we believe that antiviral treatment should be strongly considered after the end of immuno-chemotherapy, when a remission of the lymphoma has been achieved. This might also enhance duration of remission, although still to be demonstrated. Although some favorable reports exist, antiviral therapy should not be routinely associated to chemotherapy outside clinical trials, but eventually administered in the form of ribavirin when symptomatic flares occur.

In conclusion, treatment of HCV associated DLBCL should be performed in an interdisciplinary approach with hepatologists and hematologists working hand in hand with close monitoring of liver function. This should ensure a standard curative approach to most HCV-positive DLBCL, not depriving them from the chance of being cured, since severe hepatic complications in patients with no other risk factors are rare.

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P- Reviewer: Dang SS, El-Bendary M, Schiavon LL, Vespasiani-Gentilucci U
S- Editor: Gou SX **L- Editor**: A **E- Editor**: Ma S

World Journal of Gastroenterology

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DOI: 10.3748/wjg.v20.i32.11062

World J Gastroenterol 2014 August 28; 20(32): 11062-11068
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (7): Liver transplant

Beyond the Pediatric end-stage liver disease system: Solutions for infants with biliary atresia requiring liver transplant

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Received: February 13, 2014 Revised: May 13, 2014
Accepted: June 21, 2014
Published online: August 28, 2014

Abstract

Biliary atresia (BA), a chronic progressive cholestatic disease of infants, is the leading cause for liver transplant in children, especially in patients under two years of age. BA can be successfully treated with the Kasai portoenterostomy; however most patients still require a liver transplant, with up to one half of BA children needing a transplant by age two. In the current pediatric end-stage liver disease system, children with BA face the risk of not receiving a liver in a safe and timely manner. In this review, we discuss a number of possible solutions to help these children. We focus on two general approaches: (1) preventing/delaying need for transplantation, by optimizing the success of the Kasai operation; and (2) expediting transplantation when needed, by performing techniques other than the standard deceased-donor, whole, ABO-matched organ transplant.

Key words: Biliary atresia; Liver transplantation; Pediatric liver disease; Pediatric end-stage liver disease; Kasai operation; Newborn screening; Surgical outcomes; Living-related donor transplantation; Split liver transplantation; ABO-incompatible liver transplantation

Core tip: Infants with biliary atresia (BA) can benefit by maximizing the Kasai operation's success, through diagnosing the disease earlier, employing experienced surgeons, ensuring adequate nutrition, and administering certain medications. If they require a liver transplant despite these interventions, infants with BA can also benefit by expediting the transplant process, through the use of living-related donor, split or ABO-incompatible liver transplants.

Tessier MEM, Harpavat S, Shepherd RW, Hiremath GS, Brandt ML, Fisher A, Goss JA. Beyond the Pediatric end-stage liver disease system: Solutions for infants with biliary atresia requiring liver transplant. *World J Gastroenterol* 2014; 20(32): 11062-11068 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11062.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11062

INTRODUCTION

Children in the United States requiring liver transplantation are prioritized organs according to the pediatric end-stage liver disease (PELD) scoring system. PELD estimates mortality risk in order to assign deceased donor organs to the children who need them most. PELD weighs three variables - total bilirubin, albumin, and INR - and gives special consideration to patients who are less than two years old and/or weigh two standard deviations

below normal[1]. Initially, the PELD system functioned successfully, with only 3% of children in the early PELD era (2000-2004) dying while waiting for transplant[2]. Unfortunately, in recent years, the PELD system has not been as efficacious for the youngest children, *i.e.*, those less than 2 years old. Over the past 10 years, approximately 15% of these children with chronic liver disease either died while waiting on the transplant list or were removed from the list because they became too ill[3]. Similarly, other countries using the PELD system, such as Brazil, experience similar waiting list death rates of approximately 15%[4].

A large number of these young children requiring liver transplant suffer from the same disease: biliary atresia. Biliary atresia (BA) is a progressive cholestatic and fibrotic disease of unknown etiology which occurs only in infants. Children often appear normal at birth but become jaundiced in the first weeks of life. Those diagnosed early receive a Kasai portoenterostomy, which removes the obstructed extra-hepatic bile ducts in an attempt to restore normal bile flow. Some Kasai operations are successful; however, many will fail. Children with a "failed Kasai" will require liver transplant in infancy to survive. In addition, infants who are diagnosed late often have too much liver damage to benefit from the Kasai operation and will also require an early transplant, usually in the first year of life. Unfortunately these two groups encompass anywhere from one-third to greater than one half of the BA population[5]. For example in the United States, a multicenter study observed that 46 out of 104 infants with BA either required liver transplant or died by 24 mo of age[6].

Infants with BA requiring liver transplant are especially vulnerable[7-9] as they do not fare well in the current PELD system. First, PELD does not account for acute life-threatening complications that commonly develop in BA, such as refractory ascites, acute variceal bleeds, and infections such as cholangitis. Second, PELD calculations award points to patients with low albumin and weight *Z*-scores, both of which are often artificially normal in infants with BA. Low serum albumin concentrations can be masked by infusions, and weight can be elevated by fluid overload and enhanced by parenteral nutrition. Third, PELD automatically diverts the already rare small organs away from infants with BA - no matter how high their PELD score - by allocating them to infants with metabolic or oncological disorders first.

In this review, we discuss ways to improve outcomes in infants with BA requiring liver transplant. Our focus is on interventions beyond the PELD system, and falls into two categories: (1) optimizing the success of the Kasai operation; and (2) maximizing liver transplants when needed, before BA liver disease progresses too far to make transplant unsafe.

OPTIMIZING KASAI OUTCOMES

The Kasai portoenterostomy is the only treatment for infants with BA other than liver transplant. The Kasai operation has variable outcomes, with a 53.7% and 46.7% 1- and 2-year transplant-free success rate in a North American study[10]. Hence, improving the success rate could dramatically decrease the need for liver transplants in infancy. The Kasai operation's success rate may be influenced by at least 4 factors: (1) time of Kasai; (2) surgeon experience; (3) post-operative nutrition; and (4) post-operative medications.

Early diagnosis of BA and referral for Kasai

Many studies have correlated earlier Kasai portoenterostomies with the best outcomes and reduced need for transplant[5]. For example, in one large study of 743 infants, 66% of infants receiving a Kasai portoenterostomy before 30 days of life (DoL) were transplant-free at two years, compared to 58% with Kasai performed between 45 and 60 DoL and 42% when performed at greater than 90 DoL[11]. Similarly, a North American study of 104 infants detected a trend, though not statistically significant, toward better outcomes with greatest transplant-free survival when the Kasai portoenterostomy was performed at less than 30 DoL[6]. It is important to note that all these studies demonstrate correlation rather than causality. However, given the strong correlation a more definitive randomized-controlled trial would be unethical to perform.

The main barrier to an early Kasai portoenterostomy is that BA is difficult to diagnose. In the US, we have no standard way to identify it early. The disease starts insidiously, with newborns typically appearing healthy and only developing jaundice later. Furthermore, practitioners often mistake the disease's jaundice as "physiological" or "breast milk" jaundice. In other countries such as Taiwan, infants are screened for BA with a stool color card (SCC) that parents use to detect the acholic stools characteristic of extrahepatic biliary obstruction. The SCC is an effective screen, with a sensitivity of 82.9% for detection before 45 DoL and 97.1% at 60 DoL[12].

Early detection of BA may also be achieved by measurement of newborn serum direct/conjugated bilirubin[13]. This test has a number of advantages. Newborn direct/conjugated bilirubin measurements are very sensitive for BA, as infants with the disease have high direct/conjugated bilirubin levels at birth. The test is also very specific if all abnormal levels are confirmed with a single repeat test at the two-week well child check (Harpavat S, in preparation). The test has the additional advantage of already being used in clinical practice and thus does not require any additional infrastructure or training. Newborn bilirubin screening is now being tested prospectively to determine whether it could be a standard-of-care test in the newborn nursery to help detect BA earlier.

Enhancing surgical outcomes via experience

Because BA has an incidence of only one in 8000 to 18000 infants[5,10], few surgeons have the opportunity to perform multiple Kasai operations. Several studies have

suggested that successful outcomes for the Kasai are dependent on the experience of the operative surgeon and the presence of an organized team of medical and surgical personnel to care for children with BA[5,14,15]. Studies in the United Kingdom demonstrated a clear distinction in jaundice-free two-year survival among high activity centers, with those performing > 5 Kasai portoenterostomies/year enjoying 43% success, those performing 2-5 Kasais/year 29% success, and those performing 1 Kasai/year only 11% success[14]. As a result of the study, all Kasai portoenterostomies in the United Kingdom are now performed in large volume centers. Similar trends were found in high *vs* low volume centers in France, which spurred a collaborative initiative among centers in an attempt to equalize outcomes[15].

Surgeons performing the Kasai operation face a number of technical challenges which may explain why those most experienced have the greatest success. There are many anatomical variants in BA, leading to different appearances of the central, distal, and proximal biliary trees[10]; experience with these variations certainly can improve outcomes. Surgeons also vary in how deep into the hilum they dissect when removing the proximal portions of the obstructed duct. Whether a deeper dissection improves outcomes remains a topic for further study[16]. Furthermore, after removing the obstruction, surgeons can connect the liver to intestine in various ways. For example, some were previously attaching the intestine to a patent gallbladder before this technique was proven inefficacious[10].

Aggressive post-Kasai nutrition

Nutrition is vital in BA patients post-Kasai; as with many chronic diseases, there can be both increased caloric demands and decreased oral intake in patients with BA[17,18] Adequate nutrition correlates with both improved post-Kasai and post-transplant outcomes. In a study of 100 infants, those surviving two years jaundice-free with their native liver had higher weight and length *Z*-scores when compared to those requiring liver transplant[19]. The differences in weight and height were statistically significant 6 and 18 mo after the Kasai operation, respectively; however, it is important to note that both groups had lower weight and length *Z*-scores when compared to the general population[19]. Additionally, better nutrition at time of transplant is associated with decreased mortality post-transplant[18].

Aggressive nutritional therapy is thus warranted as it may improve both post-Kasai and post-transplant outcomes. While there are no validated nutrition protocols for infants with BA, we follow a protocol based on a number of parameters. First, infants receive nutrition in the first 24 h after the Kasai procedure. This often involves temporary parenteral nutrition while awaiting resumption of gut function. Second, infants are started on enteral feeds of breast milk supplemented with medium chain triglycerides (MCT) or MCT-based formulas, to achieve 120-150 kcal/kg per day. However, infants are often unable to take this increased volume orally, as cholestasis can contribute to poor appetite[20], and require naso-gastric feeds to meet these goals. Third, if infants are still unable to gain weight with enteral feeds, parenteral nutrition is started. Parenteral nutrition both increases the nutritional status and equalizes outcomes post-transplant of malnourished BA patients when compared to their better-nourished counterparts[21]. Fourth, infants receive high doses of fat-soluble vitamins in an attempt to increase vitamin D, A and E levels, as well as to help maintain normal clotting parameters[22].

Post-operative medications

Several medications are administered post-Kasai to improve outcomes, with variable success and data to support their use. Antibiotics are typically given following surgery to prevent gut bacteria from entering the intrahepatic biliary tree and causing cholangitis. A prospective randomized study found that patients receiving either sulfamethoxazole/trimethoprim (average 14.6 mo treatment) or neomycin (average 14.7 mo treatment) had decreased rates of cholangitis and increased survival when compared to historical controls[23]. Another study detected a trend towards improved two year liver transplant-free survival if antibiotics were given for more than three months after Kasai; however, interestingly, the rates of cholangitis were statistically no different between groups of children receiving antibiotics (55%) and those not (49%)[6].

Ursodiol is also commonly prescribed and continued indefinitely, usually at a dose of 15-30 mg/kg per day[6]. Ursodiol is a naturally-occurring bile acid thought to help flush bile through ducts and out of the liver. Ursodiol has a suggested benefit in several observational and case-control studies, including one in which sixteen 18-mo old post-Kasai BA children stopped Ursodiol for 3 mo. Prior to discontinuation, their mean dose of Ursodiol was 25 mg/kg per day (range: 20-36 mg/kg per day). Twelve of these children had worsened liver panels, and one child developed jaundice when Ursodiol was discontinued[24]. Not all trials, however, support Ursodiol use. Other studies report no benefit and perhaps even harm with Ursodiol at a dose of 20 mg/kg per day[25]. A prospective randomized controlled trial for Ursodiol would better assess the drug's benefit.

Steroids are the most tested, and the most controversial, of the post-Kasai medications. Steroids are used because many consider the progressive fibrosis of BA to be due to an inflammatory immune-mediated process[26]. Corticosteroids were recently evaluated in the large double-blind randomized placebo-controlled START trial of 140 infants. Corticosteroids were given to 70 infants after the Kasai operation, at a daily dose of 4 mg/kg *iv* methylprednisolone for two weeks, followed by 2 mg/kg oral prednisolone for two weeks, followed by a taper over the next nine weeks. Compared to the 70 control infants receiving placebo, the experimental subjects had equivalent rates of restored bile flow at six months and two

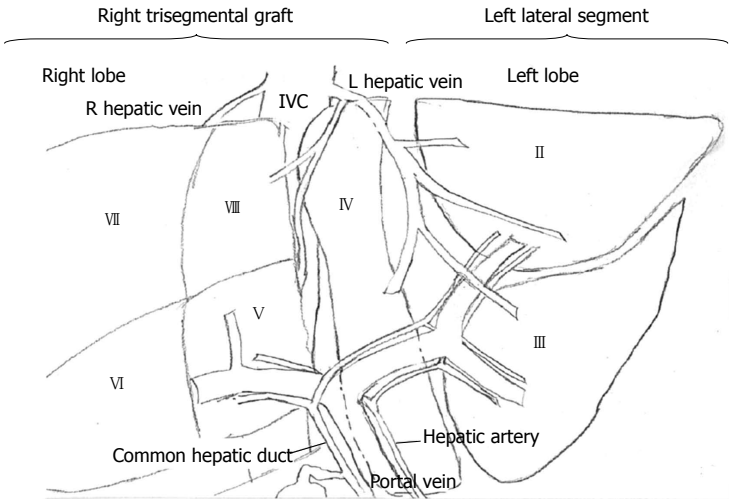

Figure 1 Diagram of split liver transplant. The left lateral segment is donated to a child and the right trisegmental graft is designated for an adult.

years, as well as similar rates of liver transplant-free survival at two years. However, those receiving steroids had a shorter time to first serious adverse event compared to controls[27].

In part because of this trial we do not prescribe steroids, though many practitioners do because of potential benefits previously reported. An earlier two-center, double-blind, randomized, placebo-controlled trial showed that steroids reduced serum bilirubin levels without improving transplant-free survival[28]. An additional follow-up study demonstrated decreased bilirubin levels with high dose (5 mg/kg per day) *vs* low dose (2 mg/kg per day) corticosteroids, but again demonstrated no improved survival effect[29]. Recently a study from Japan also reported decreased bilirubin levels with high (4 mg/kg per day) *vs* low (2 mg/kg per day) dose corticosteroids[30]. However, they did not report improvements in transplant-free survival.

MAXIMIZING TRANSPLANT OPTIONS FOR CHILDREN WITH BA-ASSOCIATED LIVER FAILURE

As mentioned above, infants with BA who need a liver transplant will often have low natural PELD scores. To circumvent this, infants must earn "exception points," granted to them at the request of their physicians by an anonymous board reviewing their case. Still, even with these exception points, many infants receive a transplant only after long waits, worsening disease, and deteriorating health. There are at least three ways for infants to avoid these delays and expedite liver transplant: (1) performing living-related donor (LRD) liver transplants; (2) increasing "split" transplants; and (3) permitting ABO-incompatible transplants (ILTs).

Living-related donor transplants

Living-related donor (LRD) transplants have excellent outcomes. In Japan, where deceased-donor liver transplants are not always accepted culturally, LRD transplants are standard with 88.3% one-year, 85.4% 5-year, 82.8% 10-year and 79.6% 20-year survival rates[7]. Survival for BA, which accounted for 66% of all Japanese pediatric transplants, was just as successful for LRD with a 20-year survival rate of 84%[7]. LRD transplants are especially advantageous because they can occur when they are most likely to result in the best outcome: when transplant is needed but before the infant becomes too ill to safely tolerate the operation. In contrast, with deceased donor transplants, infants with BA can spend many months waiting for a suitable organ to become available and must compete with other infants in the PELD system[31].

Despite these good outcomes, LRD transplants do pose a number of challenges. First, LRD transplants raise ethical issues regarding the choice of donor. It is unclear who should be considered, *i.e.*, only parents *vs* extended family *vs* others. Second, LRD does pose a risk to those caregivers most vital to the care of an infant after transplant. If a patient's parent decides to donate and then suffers from operative complications, the infant may no longer have stable support for further care. Third, in the US, LRD transplants create a "two-tier" system of transplant because public insurers are less likely to cover the donor's surgery[31]. As a result, LRD raises the prospect of privately-insured infants with BA surviving longer than publically-insured infants because the former are able to receive an earlier transplant. In our experience, this insurance issue precludes many of our BA patients from qualifying for a LRD transplant.

Increasing "split" liver transplants

"Split" liver transplants maximize the deceased-donor organs available by allowing for one donor to benefit two recipients. The procedure involves splitting the liver into a left lateral segment and a right trisegmental graft, with the smaller left lateral segment allocated to a child (Figure 1). "Split" transplants are technically more challenging,

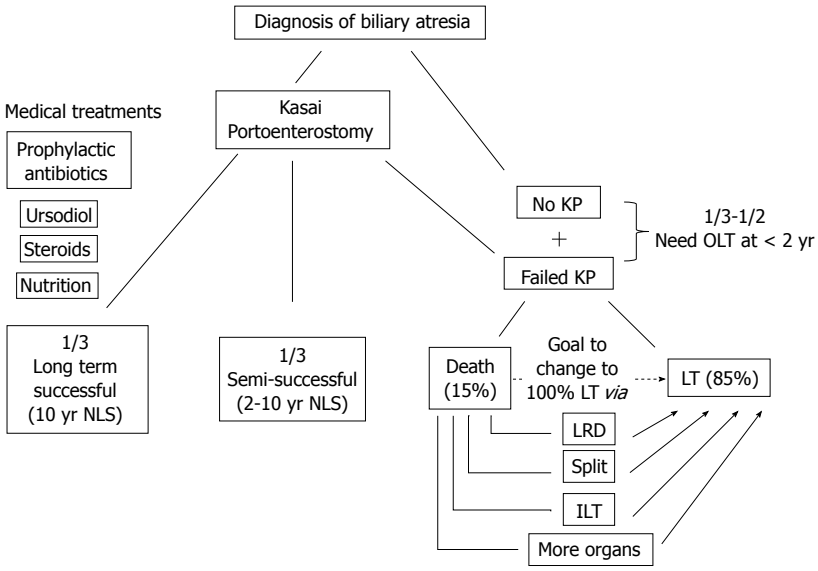

Figure 2 Outcomes in the current era of biliary atresia and ways to improve patient outcomes. KP: Kasai portoenterostomy; NLS: Native liver survival; LT: Liver transplant; LRD: Living related donor; ILT: Incompatible donor liver transplant.

and in previous eras, split transplants had more complications when compared to whole graft transplants[32]. However, recently these differences have been resolved, with no significant survival differences between whole and split liver transplants. Both the children and adults receiving left lateral segments and right trisegmental grafts, respectively, have the same low rates of graft failure and mortality when compared to those patients receiving whole organs[32,33].

Unfortunately, at this time, less than 10% of donor liver that could be split are split[32]. This may be due to a number of factors, including surgeon comfort with the technique. Another barrier is that recipients currently decide whether they prefer a split or whole liver, with most recipients opting for a whole liver. To address this, the OPTN/UNOS Ethics committee now suggests that patients not be given this choice in centers where split liver transplants have equal outcomes to whole liver transplants. Their White Paper statement endorses "splitting these optimum livers should be considered the benchmark rather than the exception. Fostering maximum utilization of these organs is ethically proper and should be required"[34]. This has the potential to significantly improve organ availability for young infants with BA. However, these are only recommendations, and a major avenue for future advocacy should be in converting these recommendations into formal policy.

ABO-incompatible liver transplants

ABO-incompatible donor liver transplants (ILT) represent one of the next frontiers of pediatric liver transplant. Traditionally, matching a donor liver to recipient is based solely on ABO compatibility, unlike heart and kidney transplantations which also rely on HLA-typing[35,36]. However, good results have been demonstrated with ILT, which questions the need for ABO-compatibility in children. A 2011 study in the United Kingdom demonstrated equal outcomes in 5 infant ILT recipients when compared to 25 infants who received ABO-compatible liver transplants (CLT); there was no difference in rejection, biliary complications or patient survival after an average of 3 years[37]. Additionally, a 2011 meta-analysis encompassing greater than 40 years of over 3500 pediatric and adult ILT patients around the world showed no difference in 1-, 3-, 5- and 10-year outcomes in pediatric ILT patients when compared to pediatric CLT[38]. Similarly, in Japan, ILT survival in 185 recipients transplanted at less than 2 years of age was 81% at 15 years, further validating the safety of ILT[7]. Given the outcomes described and the need for organs for the sickest pediatric liver patients, especially patients with BA, ABO-incompatible liver transplantation may be another way to limit waiting list mortality.

CONCLUSION

Infants with chronic liver failure from BA are a vulnerable group; the natural history of their disease course is grim. The Kasai portoenterostomy can result in excellent outcomes in a small subset of children with BA, with resulting normal liver function and quality of life for decades. However, the majority of children with BA will ultimately require transplantation, and infants with BA who need a liver transplant are particularly at risk. These infants wait on the transplant list based on their PELD score, while their clinical condition invariably worsens. Fortunate infants are matched with a deceased-donor organ in a timely fashion and often have excellent outcomes. However, too many infants with BA receive a liver transplant later than ideal or never have the chance to receive one at all. To help these infants, practitioners spend considerable energy applying for PELD "exception points" and extending life by managing serious complications as they arise. We suggest that practitioners could serve infants

with BA better (Figure 2) with two additional interventions: (1) preventing/delaying need for liver transplant, by optimizing the success of Kasai operation; and (2) maximizing the availability of liver transplants when needed, through LRD transplants, "split" transplants, and ILTs.

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P- Reviewer: Boucek C **S- Editor**: Qi Y **L- Editor**: A
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DOI: 10.3748/wjg.v20.i32.11069

World J Gastroenterol 2014 August 28; 20(32): 11069-11079
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (7): Liver transplant

Natural history, treatment and prevention of hepatitis C recurrence after liver transplantation: Past, present and future

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Received: October 29, 2013 Revised: March 7, 2014
Accepted: June 20, 2014
Published online: August 28, 2014

Core tip: This paper reviews the evolution of our knowledge on the natural history of hepatitis C virus (HCV) recurrence after liver transplantation, including risk factors for disease progression, and antiviral therapy. It is necessary to define an innovative public health policy to improve HCV screening which is the only way of allowing non-tested HCV patients access to therapy before they develop advanced liver disease.

Dumortier J, Boillot O, Scoazec JY. Natural history, treatment and prevention of hepatitis C recurrence after liver transplantation: Past, present and future. *World J Gastroenterol* 2014; 20(32): 11069-11079 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11069.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11069

Abstract

Hepatitis C virus (HCV)-related liver disease, including cirrhosis and hepatocellular carcinoma is the main indication for liver transplantation (LT) worldwide. Post-transplant HCV re-infection is almost universal and results in accelerated progression from acute hepatitis to chronic hepatitis, and liver cirrhosis. Comprehension and treatment of recurrent HCV infection after LT have been major issues for all transplant hepatologists and transplant surgeons for the last decades. The aim of this paper is to review the evolution of our knowledge on the natural history of HCV recurrence after LT, including risk factors for disease progression, and antiviral therapy. We will focus our attention on possible ways (present and future) to improve the final long-term results of LT for HCV-related liver disease.

Key words: Hepatitis C; Liver transplantation; Recurrence; Fibrosis; Treatment

INTRODUCTION

Hepatitis C virus (HCV) has been estimated to infect 170 million people worldwide[1]. Liver disease due to HCV, including cirrhosis and hepatocellular carcinoma (HCC) is the main indication for liver transplantation (LT)[2,3]. Post-transplant HCV re-infection of the graft is reported to be universal and often results in accelerated progression from acute hepatitis to chronic hepatitis, and liver cirrhosis. Comprehension and treatment of recurrent HCV infection after LT have been major issues for all transplant hepatologists and transplant surgeons for the last 25 years. This is illustrated by the significant number of published papers in this field to date (1050 references in PubMed up to September 2013). The aim of this paper is to review the evolution of our knowledge on the natural history of HCV recurrence after LT including risk factors for disease progression, and antiviral therapy. We will focus our attention on possible ways (present and future)

to improve the final long-term results of LT for HCV-related liver disease.

NATURAL HISTORY OF HCV RECURRENCE AFTER LT

Recurrence of HCV infection after LT is universal, however, the natural history of hepatitis C on the liver graft is variable. It must been pointed out that the evaluation of recurrent hepatitis C has been described from liver biopsy, which is the cornerstone of clinical management of patients in daily practice. Nevertheless, differentiation of recurrent hepatitis C from acute cellular rejection can be difficult. Regev *et al*[4] demonstrated, from 105 cases blindly reviewed by 5 pathologists, that histological distinction between recurrent hepatitis C and acute rejection had low interobserver and intraobserver agreement rates, and hence showed low reliability. It has been suggested that transient elastography may provide good accuracy in identifying patients with severe fibrosis, better than non-invasive indices based on clinico-serological parameters, in LT recipients[5].

Histological recurrence is observed in 80% of HCV-infected patients within 5 years after LT[6]. It is well known that liver disease caused by HCV infection progresses more rapidly in immunosuppressed than in immuno-competent individuals, and chronic HCV infection leads to cirrhosis in up to 20%-30% of individuals only five years after LT[7-9]. In addition, the natural history of HCV recurrent cirrhosis is also accelerated after LT. The rate of decompensation is > 40% at 1 year and > 70% at 3 years in LT recipients *vs* < 5% and < 10%, respectively, in immuno-competent patients[9-11]. The rate of progression from decompensation to death is also accelerated, with a 3-year survival of < 10% following the first decompensation *vs* > 60% in immuno-competent patients[9,10]. Therefore, long-term graft and patient survival is significantly reduced in patients undergoing LT for HCV-related disease as compared to other indications[12]. Disease progression (*i.e.*, fibrosis progression) depends on a large number of variables, including host, donor, viral, and external factors. Interestingly, early post-LT (1-year) liver biopsy has great prognostic value and must be performed in every LT recipient. The 5-year probability of graft cirrhosis is associated with the severity of necroinflammatory activity of recurrent hepatitis C on the liver graft at 12 mo[13-15]. Similarly, the graft survival is significantly impaired in recipients with early HCV recurrence and fibrosis stage 3 and 4 at 1 year after LT[16].

Recurrent HCV-related liver disease after LT begins with a first phase of acute hepatitis and thereafter can have two distinct clinical and histological patterns. The first one is the most frequent and is the same as that described in non-transplant, or non immuno-compromised patients, and is characterized by the progression from chronic hepatitis to cirrhosis. Earlier onset of biochemical hepatitis and persistently elevated serum transaminases are associated with more rapid progression of fibrosis[17-20]. The second type of recurrent hepatitis C after LT is specific for immunosuppressed patients (including HIV-positive patients and organ-transplanted patients, or both), and has been described as fibrosing cholestatic hepatitis (FCH). FCH has been observed in the context of hepatitis B virus or HCV infection and is characterized by rapid and extensive dense portal fibrosis and cholestasis leading to an inexorable deterioration in liver function[21,22]. Estimates of HCV-related FCH frequency after LT range from 2%-14%[23-26]. It has been hypothesized that FCH could be related to direct viral cytotoxicity after immune escape[27], associated with a deviation from a T helper 1 type intrahepatic cytokine response to a T helper 2 type response[28]. As its prognosis is very poor (50% of patients die[23]), predicting the onset of FCH and defining the patients at risk of developing this severe complication is a major goal. It has been suggested that FCH could be associated with older donor, corticosteroid treatment for acute rejection, stable quasispecies variants of HCV, earlier recurrence of HCV infection, higher HCV viral load, unfavorable recipient IL-28B polymorphism, and lower levels of immunosuppressive medications[26,27,29,30]. Retransplantation for this specific complication remains controversial regarding its usual poor outcome[31].

Besides FCH, many reports have identified a large number of pre-transplant and post-transplant factors associated with more rapid progression of fibrosis, without taking into account antiviral treatment. The vast majority of these studies were retrospective, and this can be explained by the necessity to have mid- and long-term (5- or 10-year) evaluation of the liver graft. Donor features are major non-viral factors which are strongly associated with fibrosis progression. Older age, liver steatosis and diabetes mellitus are all deleterious[16,32-37]. Donor age is probably the most relevant factor, but significant cut-offs (defining "old" donor) range from 33 to 50 years old. In addition, hepatitis C recurrence after LT from a donor older than 60 years could be a major factor in female recipients compared to male recipients[38]. During the past 20 years, donor age has considerably increased (mean age in France was 53.2 in 2012, with 42% of the donors over 60, from the annual report of French Agence de la Biomédecine) and the selection of young donors for HCV recipients is questionable. The role of recipient age is still debated. Selzner *et al*[39] evaluated the role of donor and recipient age in transplantation/ischemia-reperfusion injury and short- and long-term graft and patient survival in a large population of 822 LT recipients. The HCV patients who were ≥ 50 years old and who were transplanted with an older graft (≥ 60 years) had significantly reduced 3- and 5-year graft survival. Interestingly, the use of HCV-positive donors has been reported not to alter the outcome after LT, and this is a way to enhance the number of available liver grafts[40]. Recently, it has been suggested that the use of HCV-positive liver grafts from donors > 45 years could be associated with more advanced fibrosis[41]. At the time of LT, it has been suggested that prolonged cold and/or warm ischemia times

could negatively impact graft and patient survival[42]. Some recipient characteristics also have a significant impact on HCV recurrence after LT. The reported impact of IL-28B genotypes (donor and recipient) on progression to cirrhosis after hepatitis C recurrence has been inconsistent[43]. Reactivation of herpes group viruses [cytomegalovirus (CMV) and human herpes virus-6 (HHV-6)] may play a role in HCV recurrence after LT. Some reports have suggested that herpes virus infection could accelerate fibrosis progression[44,45]. Recently, a retrospective study of 347 first LT recipients showed that CMV infection was associated with an increased risk of fibrosis stage $\geqslant$ 2 and inflammation grade $\geqslant$ 2[46]. However, the widespread use of highly effective CMV prophylaxis regimens has probably reduced any potential effect of herpes virus reactivation on recurrent hepatitis C during the last decade. The risk of *de novo* diabetes mellitus after LT is increased in patients with HCV infection, especially in patients treated with tacrolimus (Tac)[47], and the presence of diabetes is associated with more rapid fibrosis progression[48]. Similarly, alcohol consumption after LT also acts as co-factor and accelerates fibrosis progression in HCV patients[49]. Some studies have suggested that HLA class I and II matching could increase fibrosis progression, without interfering with rejection[50,51]. The prevalence of cryoglobulinemia is about 15%-20% in patients with end-stage HCV-related liver disease and almost 30% in LT recipients with recurrent hepatitis C. This has been found to be associated with reduced graft survival from more severe HCV recurrence and increased incidence of hepatic artery thrombosis[52-54]. In summary, regarding the potential deleterious effect of all of these donor/recipient characteristics, from which a vast majority is not modifiable, the use a non steatotic liver graft from a young donor (< 50 years) could be recommended to reduce cold ischemia time, and to actively prevent CMV reactivation or primo-infection in each case of HCV LT recipient.

In addition to donor/recipient characteristics, the second major factor which can modify the natural history of recurrent hepatitis C after LT is HCV itself. First of all, higher HCV RNA levels (in serum and/or liver) at the time of LT are associated with increased risk of progression to cirrhosis, graft loss, and death[55,56]; higher viral load in the early post-transplant period has also been associated with more rapid progression[57]. The data on the relationship between HCV genotype and recurrent hepatitis C infection are more conflicting, and the impact of response to antiviral treatment induces major interferences. Nevertheless, genotypes 1b and 4 could be associated with more severe recurrent hepatitis C[13,58-61]. During the last decade, human immunodeficiency virus (HIV)-HCV co-infection has emerged as a new indication for LT because of the major progress in the treatment of HIV infection. Duclos-Vallée *et al*[62] first compared the survival and severity of recurrent HCV infection after LT in 35 HIV-HCV-co-infected and 44 HCV-monoinfected patients. The 2-year and 5-year survival rates were significantly reduced in co-infected patients: 73% and 51% *vs* 91% and 81% in monoinfected patients, respectively. The progression of fibrosis was significantly higher in the co-infected group. Therefore, improvement of prognosis in this specific indication is a major challenging issue for LT hepatologists.

Finally, the third goal in the field of transplantation is the impact of the immunosuppressive regimen on the severity of HCV recurrence. The effects of steroids on recurrent hepatitis C are complex. High-dose intravenous steroid boluses for acute rejection lead to a significant increase in HCV viral load and are associated with more rapid progression of recurrent hepatitis C[55]. In addition, adjuvant antibody therapy (anti-thymocyte globulins, OKT3) for steroid-resistant rejection may be associated with more rapid progression to severe fibrosis[63,64]. More recent data suggest that maintaining low-dose steroids over a long period could have beneficial effects on the progression of recurrent hepatitis C[65-67] and the steroid-free regimen did not show an advantage in HCV recurrence[68]. Similarly, the long-term use of azathioprine as part of maintenance immunosuppression therapy may be beneficial[20,66,69]. The role of calcineurin inhibitors (CNIs), Tac and cyclosporine (CsA), on the severity of recurrent hepatitis C after LT is highly controversial. CsA might offer potential advantages over Tac. First, CsA directly suppresses HCV replication *in vitro* by binding to cyclophilin B and inhibiting HCV RNA polymerase[70]. Moreover, Tac, but not CsA, indirectly enhances HCV replication *in vitro* through inhibition of phosphorylation and nuclear translocation of STAT-1, thereby blocking interferon signaling pathways[71]. These experimental data could explain the findings of some retrospective studies which showed lower progression of fibrosis in patients receiving CsA instead of Tac[32,69,72]. Nevertheless, several prospective randomized controlled studies comparing CsA and Tac as primary immunosuppressive drugs in HCV LT recipients have observed no differences in liver fibrosis and graft or patient survival[73-76]. These results must be interpreted with caution since all endpoints measurement were probably too premature (< 5 years) to reach clinical relevance, especially in patients who received antiviral treatment. The influence of mTOR inhibitors requires further attention, since their inhibitory effects on transforming growth factor β and procollagen will delay liver graft fibrosis in LT patients with recurrent hepatitis C[77]. In summary, regarding the lack of evidence regarding the potential role of immunosuppressive regimens on HCV recurrence after LT, it is recommended that the use of high doses of steroids and/or anti-thymocyte globulins or OKT3 for the treatment of acute rejection should be avoided.

TREATMENT OF HCV RECURRENCE AFTER LT: PAST AND PRESENT

The available evidence suggests that the best way to improve long-term outcome after LT for HCV-related

liver disease is to cure HCV infection. For this purpose, three different strategies have been evaluated: (1) antiviral treatment before LT; (2) early antiviral (pre-emptive) treatment after LT; and (3) antiviral treatment at the time of biopsy-proven recurrent hepatitis C on liver graft. The majority of relevant available data concerns antiviral combination therapy using pegylated alpha-interferon (PegIFN) and ribavirin (RBV).

The limits of antiviral treatment in patients awaiting LT include reduced efficacy in cirrhotic patients, the necessity to maintain the treatment for a relatively long period of time (in case of virological response), from 6 to 12 mo, problems in achieving full doses of PegIFN and RBV due to side effects (mainly hematological), and the risk of severe, sometimes lethal, complications (mainly infectious), especially in patients with decompensated cirrhosis[78]. The best candidates for therapy are Child-Pugh class A patients, with a relatively long expected time before LT (patients with HCC), especially in patients with a non-1 genotype, and the treatment should be maintained only in the case of early (or rapid) virological response[79]. The first generation direct acting antivirals (DAA), telaprevir and boceprevir, have not dramatically modified this algorithm, as better efficacy is counterbalanced by the high risk of severe infectious events in compensated cirrhotic patients[80]. Similarly, early (first month post-LT) pre-emptive antiviral treatment is usually not feasible and associated with poor hematological tolerance[81-85]. As a result, the vast majority of patients receive antiviral therapy when recurrent HCV hepatitis on liver graft is established, usually at least one year after LT. With PegIFN and RBV, the reported rate of viral clearance ranged from 20%-48%[86-92]. Factors associated with lower response when compared to non-LT patients include high viral load and high prevalence of genotype 1. Older donor age may also hinder the success of post-transplantation antiviral therapy[93-95]. Moreover, dose reduction and discontinuation of treatment were common in all studies (approximately 75% and 25%, respectively), due to adverse events and possibly represented the most important obstacles to attainment of viral clearance[96]. Interestingly, RBV dose reduction due to renal impairment probably has lower clinical impact as efficient plasma RBV concentrations can be obtained[97]. In addition, acute rejection often occurred during treatment (especially when protocol biopsies were performed[98]), which can induce graft dysfunction, and in some rare cases, graft failure and patient death[91]. Finally, it has been suggested that CsA may increase the antiviral effect of interferon-based therapy, when compared to Tac, by reducing the rate of relapse after an end-of treatment viral clearance[92,99-101]. The use of telaprevir and boceprevir after LT is a challenge, due to potential toxicity and drug-drug interactions with CNIs[102]. The first experience from 9 patients suggested that telaprevir-based triple therapy could be highly effective in LT patients, and that drug-drug interactions between telaprevir and immunosuppressants could be handled appropriately by the close monitoring of trough levels and adequate dosage adjustments[103]. Further experience has recently been reported in a US cohort of 60 patients and in a French cohort of 37 patients[104,105]. In the French study, the end of treatment virological response rate was 72% in the boceprevir group and 40% in the telaprevir group[105]. When used with boceprevir, CsA dose was reduced by 36% and Tac dose by 78%; when used with telaprevir, CsA dose was reduced by 48% and Tac dose was reduced by 95%. Infections occurred in 27% of patients, with a fatal outcome in one third. The most common adverse effect was anemia (92%), treated with erythropoietin and/or RBV dose reduction; 35% of the patients received red blood cell transfusions. These preliminary results suggest that first generation triple therapy is effective in LT recipients, particularly after failure of previous treatment, but is associated with poor tolerance. In the US cohort, according to an intention-to-treat analysis, 14 of 21 telaprevir-treated patients (67%) and 10 of 22 patients who received boceprevir (45%) achieved undetectable HCV RNA at week 24 without viral breakthrough at the last follow-up[104]. These contradictory results are probably related to patient-related differences as these two cohorts report preliminary experiences in an open-label design.

The beneficial effect of antiviral treatment and viral clearance on liver fibrosis and survival is intuitive and has been suggested by a randomized controlled study[90] and some uncontrolled studies[106-111]. Nevertheless, a lack of impact of HCV eradication on liver fibrosis in some other studies has also been reported[86,112-116]. This might be related to (1) an insufficient statistical power due to a small number of cases; (2) a too short period of follow-up (12-18 mo), because fibrosis improvement was probably delayed; (3) the inability of the fibrosis score to show mild improvement; and (4) interferences from other determinants of fibrosis than chronic viral hepatitis in some cases (rejection, biliary complications, …). On the other hand, it has recently been suggested that maintenance antiviral treatment might slow fibrosis progression, even in cases of persistent HCV infection[111,117].

The ultimate treatment of recurrent HCV-related cirrhosis after LT is retransplantation. A number of studies have reported poor overall survival after retransplantation for this specific indication[118-123]. Although the decision to relist these patients can be difficult, a score could help to select candidates with the best potential outcome. Recently, a score which includes donor age, serum creatinine, international normalized ratio (INR) and serum albumin at the second transplantation, recipient age at the first transplantation, and the interval between first and second transplantations, has been proposed, and requires further evaluation[124].

TREATMENT AND PREVENTION OF HCV RECURRENCE AFTER LT: FUTURE PERSPECTIVES

As LT is the ultimate step in the natural history of HCV

infection, major advances in antiviral treatment would probably dramatically change the management of LT recipients in daily practice in the next decade.

The next and promising step in antiviral therapy will be the use of second generation DAA, before and after LT, with the aim of obtaining viral eradication in more than 90% of cases, in both naïve patients and non-responders to previous therapy infected with all genotypes. Nevertheless, the second generation DAA, which have recently been submitted for registration (sofosbuvir and simeprevir) still need to be combined with PegIFN and RBV in patients infected with HCV genotype 1[125]. IFN-free regimens will be the ultimate step for future therapies consisting of combinations of novel DAA which are under investigation, such as daclatasvir or asunaprevir[126,127]. The good tolerance and lack of clinically apparent drug-drug interactions with CNIs make these new DAA very attractive for future use in LT recipients[127,128]. The first case of successful IFN-free therapy (sofosbuvir and daclatasvir) in the setting of FCH has already been reported[127].

As the use of PegIFN, RBV and new DAA may be limited by resistance, adverse effects, and high costs, the development of new alternative preventive and/or therapeutic antiviral strategies is highly relevant. For example, HCV entry into hepatocytes is required for initiation, spread, and maintenance of infection, and could be the target of novel drugs, such as neutralizing antibodies for HCV envelope glycoproteins, blocking antibodies specific for host factors, or small molecular compounds against host factors or viral proteins[129-137]. Such strategies would be highly effective in the field of LT in order to prevent graft infection, and could be associated with other antiviral therapies.

Finally, emerging HCV treatments will impact on morbidity and mortality, and therefore, candidates for LT (presenting with HCC and/or decompensated cirrhosis) might significantly decrease during the next decades. Deuffic-Burban *et al*[138] recently simulated the progression of yearly-HCV-infected cohorts from the beginning of the epidemic and calculated 2013-2022 candidates for LT up to 2022 without and with therapies, in France. Overall, current treatment would enable an 88% and 42% reduction in the gap between LT and HCC and decompensated cirrhosis candidates, respectively. Interestingly, although HCV infection is treated with the same therapies in different countries, the effects of these therapies on morbidity and mortality vary significantly, suggesting that there is a need for public health policies based on population-guided therapy, in addition to common guidelines based on virological response-guided therapy[139].

In conclusion, regarding current major progress in HCV treatment, we hope that HCV-related liver disease will become a marginal indication for LT in one or two decades. Thus, as an important proportion of HCV patients are unaware of their condition, it is necessary to define an innovative public health policy to improve HCV screening, which is the only way of allowing non-tested HCV patients access to therapy before they develop advanced liver disease.

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P- Reviewer: Berg CP, Chau GY **S- Editor**: Qi Y
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DOI: 10.3748/wjg.v20.i32.11080

World J Gastroenterol 2014 August 28; 20(32): 11080-11094
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (7): Liver transplant

Postoperative biliary adverse events following orthotopic liver transplantation: Assessment with magnetic resonance cholangiography

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Received: October 19, 2013 Revised: May 11, 2014
Accepted: May 23, 2014
Published online: August 28, 2014

Abstract

Biliary adverse events following orthotopic liver transplantation (OLT) are relatively common and continue to be serious causes of morbidity, mortality, and transplant dysfunction or failure. The development of these adverse events is heavily influenced by the type of anastomosis during surgery. The low specificity of clinical and biologic findings makes the diagnosis challenging. Moreover, direct cholangiographic procedures such as endoscopic retrograde cholangiopancreatography and percutaneous transhepatic cholangiography present an inadmissible rate of adverse events to be utilized in clinically low suspected patients. Magnetic resonance (MR) maging with MR cholangiopancreatography is crucial in assessing abnormalities in the biliary system after liver surgery, including liver transplant. MR cholangiopancreatography is a safe, rapid, non-invasive, and effective diagnostic procedure for the evaluation of biliary adverse events after liver transplantation, since it plays an increasingly important role in the diagnosis and management of these events. On the basis of a recent systematic review of the literature the summary estimates of sensitivity and specificity of MR cholangiopancreatography for diagnosis of biliary adverse events following OLT were 0.95 and 0.92, respectively. It can provide a non-invasive method of imaging surgical reconstruction of the biliary anastomoses as well as adverse events including anastomotic and non-anastomotic strictures, biliary lithiasis and sphincter of Oddi dysfunction in liver transplant recipients. Nevertheless, conventional T2-weighted MR cholangiography can be implemented with T1-weighted contrast-enhanced MR cholangiography using hepatobiliary contrast agents (in particular using Gd-EOB-DTPA) in order to improve the diagnostic accuracy in the adverse events' detection such as bile leakage and strictures, especially in selected patients with biliary-enteric anastomosis.

Key words: Liver transplantation; Bile ducts; Biliary adverse events; Magnetic resonance cholangiography; Contrast-enhanced magnetic resonance cholangiography

Core tip: Biliary adverse events continue to be serious causes of morbidity, mortality, and transplant dysfunction or failure after orthotopic liver transplantation. In this article, we review the technique as well as the diagnostic role of magnetic resonance (MR) imaging with cholangiopancreatographic sequences in the assessment of adverse events following orthotopic liver transplantation. The features of the main types of biliary adverse events on MR cholangiopancreatography are presented and the recently developed techniques are also discussed in this setting, according to the biliary reconstruction and liver transplant procedure performed.

Boraschi P, Donati F. Postoperative biliary adverse events following orthotopic liver transplantation: Assessment with magnetic resonance cholangiography. *World J Gastroenterol* 2014; 20(32): 11080-11094 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11080.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11080

INTRODUCTION

Orthotopic liver transplantation (OLT) has become an accepted therapy for acute and chronic end-stage liver disease[1,2]. Today, liver transplant patients have a 5-year survival rate of approximately 75%. The improvement in survival can be attributed to better patient selection and preparation, advances in organ preservation, improved immunosuppressive therapy agents, and refinement of surgical techniques[2,3].

Patients candidates for OLT are those with fulminant liver failure or with hepatic chronic diseases in which conventional therapies are no longer effective. In patients with fulminant hepatic failure (prevalently affecting young people) a large portion of the liver parenchyma is quickly destroyed and this causes liver dysfunction, infections, hepatic encephalopathy, and acute renal failure. Chronic hepatitis B and C, autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, alcoholic liver disease, and liver injury induced by drugs are the most common chronic liver diseases that may be treated by transplantation. Patients affected by disorders of metal metabolism such as hemochromatosis and Wilson disease also may benefit from liver transplant.

BILIARY ADVERSE EVENTS AFTER ORTHOTOPIC LIVER TRANSPLANTS

Although over the years there has been a continuous improvement in survival after OLT, a significant number of adverse events is still reported. One of the most important complications following OLT is acute rejection whose diagnosis is generally obtained by graft biopsy and histologic examination. The other main adverse events are represented by arterial and venous stenosis and thrombosis; biliary strictures, lithiasis and leak; hepatic abscesses; right adrenal gland hemorrhage; fluid collections and hematomas; hepatitis virus C infection; lymphoprolifeative disease and tumors. Imaging techniques play a central role in excluding the other adverse events that may have clinical signs and symptoms as those of acute rejection[4,5].

Biliary tract adverse events are the most common complications after OLT and remain a major source of morbidity in liver transplant patients, with an incidence of 5%-32%. Adverse events such as bile leaks, anastomotic and non-anastomotic strictures, biliary stones, sludge and casts are encountered more commonly as a result of increased number of liver transplantations and the prolonged survival of transplant patients[6]. Early adverse events are those occurring within three months, whereas the late ones can be observed a few months to several years after OLT.

A major determinant of biliary adverse events' risk after OLT is represented by the choice of biliary anastomosis[7,8]. Choledochocholedochostomy (CC) and biliodigestive or choledochojejunostomy (CJ) are the most frequent types of surgical reconstruction. The first technique is a duct-to-duct anastomosis between donor and recipient choledochal ducts. Since it is simple, physiologic and allows preservation of the sphincter of Oddi, this biliary anastomosis is performed in most of liver transplant patients. On the other hand, the biliodigestive technique is an anastomosis between the donor choledochal duct and a jejunal loop of the recipient and is used in selected recipients. Since infectious colonization of the biliary tract is possible, episodes of cholangitis are reported in the natural history of these patients.

The choice of biliary reconstruction can be determined by the underlying hepatic disease, the caliber of donor and recipient choledochal ducts, previous transplant or hepato-biliary surgery and the preference of the performing surgeon. However, there are no clear guidelines on the preferred type of surgical anastomosis[6].

DIAGNOSIS OF BILIARY ADVERSE EVENTS AFTER OLT

The clinical presentation of biliary adverse events varies considerably and can vary from an asymptomatic patient with moderate liver enzyme elevations to a septic patient with fever and hypotension due to ascending cholangitis[6,9].

The prompt diagnosis and appropriate management of these adverse events are important to ensure the survival of both the organ and the patient after OLT, and the diagnostic algorithm has been repeatedly revised in order to achieve the most accurate approach[2,9-11].

Whenever a biliary adverse event is suspected, diagnostic work-up usually begins with laboratory evaluation and an abdominal Doppler ultrasound (US) that allows for the evaluation of the biliary tree and the hepatic vasculature. The positive predictive value of abdominal ultrasound is very high, especially in the presence of dilated bile ducts. In the absence of dilated bile ducts, the sensitivity of the ultrasound for detecting biliary obstruction ranges from 38% to 68%[12]. Although ultrasound is a non-invasive method to assess adverse events in recipients, a normal US examination cannot exclude the presence of biliary strictures, bile leakage or widespread abnormalities of the bile ducts[2,13,14]. When US findings are indeterminate or there is persistent clinical suspicion for an abnormality, multi-detector computed tomography (MD-CT) is often performed in the period after transplantation. This imaging technique is a fast, reliable, and non-invasive mean of visualizing hepatic artery, portal vein, hepatic veins, and inferior vena cava and assess-

ing non-vascular graft complications and extra-hepatic organs. As concerns as biliary adverse events, CT can be utilized to identify biliary obstruction or leakage, but its true role has yet to be definitely established. Recent developments in imaging technology have enabled to obtain drip infusion cholangiography with CT, that allows detailed evaluation of biliary anatomy thanks to the high resolution of images, though the availability of intravenous cholangiographic contrast media is limited to a few countries.

A more conclusive evaluation of biliary adverse events can be obtained using T-tube cholangiography or invasive procedures, such as endoscopic retrograde cholangiopancreatography (ERCP) or percutaneous trans-hepatic cholangiography (PTC)[4]. In patients with a suspicion of biliary adverse events in the early phase after OLT during which the T-tube is still in place, T-tube cholangiography is the examination of choice[15]. Nevertheless, when the T-tube is removed three months after transplantation or in the case it is not utilized at all, direct visualization of the biliary tract is only possible when using invasive procedures such as PTC and ERCP, which are associated with adverse events in 3.4% of PTC and 1%-7% of ERCP[4,15]. Although a commonly effective procedure adverse events caused by ERCP are well known and mainly include pancreatitis, gastrointestinal haemorrhage, cholangitis, hemobilia, and duodenal perforation; they can be life-threatening and can delay or even diminish the chance of managing the primary disease. Furthermore, in patients with biliodigestive anastomoses ERCP may be impossible or very difficult since endoscopic approach of the biliary tract is generally precluded because technically challenging[15-17].

Endoscopic ultrasound-guided biliary drainage (EUS-BD) is a recently introduced procedure which has been quickly accepted in recent years as a possible alternative for biliary drainage in patients in whom ERCP has previously failed[18]. Though EUS-BD is rapidly gaining popularity and attraction in the endoscopic world, the methods and indications have yet to be fully standardized and this new approach cannot be considered a treatment of first instance[18].

With the introduction of magnetic resonance cholangiopancreatography (MRCP) the same level of imaging of invasive procedures can be obtained non-invasively and this approach is particularly useful in recipients in which the tube T has been removed or has never been placed.

MR CHOLANGIOGRAPHY

As a non-invasive and accurate alternative to direct cholangiography, MR cholangiography represents the next step in the event that ultrasound does not reveal evidence of bile duct abnormalities despite clinical suspicion, and actually plays a crucial role in the assessment of biliary abnormalities after surgery[19]. Although various modifications of this technique have been recently proposed, they all require the acquisition of a heavily T2-weighted sequence, which allows to visualize the structures containing stationary or slow-moving fluids as very hyperintense areas. The quality of MRC has been significantly improved with the recent introduction of multiple three-dimensional (3D) pulse sequences. After preliminary acquisition of T1- and T2-weighted sequences in the axial plane, two MR cholangiographic techniques are conventionally performed: respiratory-triggered, thin-collimation (2.4 mm thk/-1.2 mm) 3D FRFSE T2-weighted sequences in the coronal plane and breath-hold, thick-collimation (40-60 mm), single-shot fast spin-echo T2-weighted sequences utilizing coronal/coronal oblique projections[19,20].

Very encouraging results have been reported by different authors as concerns as the MRC evaluation of biliary adverse events in patients who have undergone OLT[15,20-25]. In a recent meta-analysis published by Jorgensen *et al*[26], the authors concluded that using MRCP we can obtain an excellent diagnostic accuracy for biliary obstruction in liver transplant patients, with a combined sensitivity and specificity of 96% and 94%, respectively. On the basis of their data they also suggested that MRCP may be a suitable test in recipients having low to moderate suspicion for biliary obstruction, and the employment of this non-invasive technique may prevent the unneeded possible risks of ERCP in this clinical setting[26]. Besides, in a still more recent meta-analysis by Xu *et al*[27], these authors confirmed that MRCP is a highly accurate diagnostic technique for diagnosis of biliary complications and strictures in patients who have undergone OLT.

The disadvantages of conventional MRCP[28,29] are that it lacks functional information and so, differentiation between obstructive and non-obstructive dilatation of the bile ducts is often extremely difficult. Depiction of anatomy and lesion detection can be inadequate in a non-dilated biliary system; besides, free fluid and leak in the vicinity obscures the biliary anatomy due to overlapping[30]. Hence, there is often a need for a non-invasive imaging modality, which can provide reliable anatomic as well as functional information.

T1-weighted contrast-enhanced MR cholangiography with intravenous administration of hepato-biliary contrast agents such as Mn-DPDP, Gd-BOPTA and Gd-EOB-DTPA[31] is a technique that has been recently introduced and may provide both anatomical and functional information on the biliary tract. The above mentioned contrast media are picked up by normal hepatocytes and eliminated in the biliary system (3% to 5% for Gd-BOPTA, 20% for Mn-DPDP, 50% for Gd-EOB-DTPA)[32]. Subsequent contrast-enhanced MR imaging that can include both dynamic and delayed hepato-biliary phases are acquired by utilizing 3D breath-hold fat-suppressed T1-weighted gradient-echo imaging in the axial and coronal plane.

This emerging diagnostic tool, especially when using Gd-EOB-DTPA, is particularly helpful for depicting the anatomy of biliodigestive anastomoses and identifying

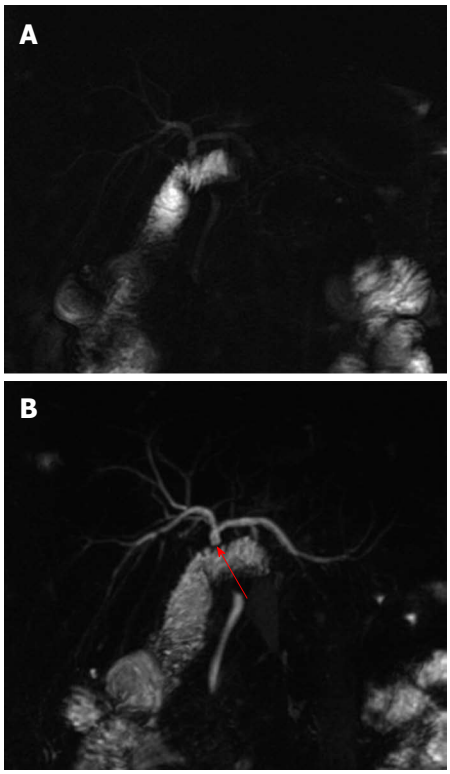

Figure 1 Bilio-enteric anastomosis. A: Single-shot thick-slab magnetic resonance cholangiogram shows a regular hepatico-jejunostomy; B: Maximum intensity projection reconstruction of 3D thin-slab fast spin-echo T2-weighted images confirms the anastomotic patency (red arrow) and better demonstrates the portion of the jejunum and the choledocho of the recipient.

adverse events such strictures of the anastomosis, bile leakages and lithiasis; besides, it can provide functional informations that are extremely promising in the grading of biliary obstruction. The drawbacks of contrast-enhanced MRC include its high cost (it is also a time-consuming technique) and its limited role in delineating the biliary tract in patients with liver dysfunction[32].

In a preliminary experience on 13 patients with hepaticojejunostomy, Hottat *et al*[29] concluded that contrast-enhanced T1-weighted MRC with intravenous administration of Mn-DPDP provides useful anatomic and functional information in patients suspected of having biliary obstruction on conventional T2-weighted MR cholangiography.

Hepatic excretion of hepato-biliary contrast agents results in enhancement of biliary structures and it is likely to have a great impact on better visualization of biliary system; on the basis of these characteristics it may potentially increase reliability of the MR examination or decrease the occurrence of a non diagnostic or equivocal interpretation[32].

BILIARY ANATOMY

In complex biliary surgical procedures, such as liver transplantation a non-invasive means of assessing the biliary tree after surgery is often necessary to exclude adverse events. MR cholangiography well depicts the postoperative reconstruction of the biliary system (particularly when it is dilated) and the different types of biliary anastomosis (Figures 1 and 2). Nevertheless, it can be limited in the visualization of the site of biliary-enteric anastomosis and also the possible cause of obstruction. Contrast-enhanced MRC may provide images with a higher resolution than those we can obtain using conventional T2-weighted MR cholangiography and has the advantage of contrast agent into the biliary system and jejunal loop, that significantly contribute to a better visualization of the anastomotic region.

BILIARY LEAKAGE

Although a series of biliary adverse events have been reported after OLT, the most frequent are leakages and strictures. The occurrence of biliary leaks is typically in the early phase after transplantation, while strictures can usually develop from several months to years[33]. Bile leaks occur in 2%-25% of cases after liver transplantation and can be classified in two categories: early bile leaks, which present within 4 wk of OLT and late bile leaks, which present beyond this time[34-37]. Early bile leaks usually occur at the anastomotic site or at the T-tube insertion site.

In liver transplant patients with both CC and a biliary-enteric anastomosis quick and correct localization of biliary leakages is helpful for guiding the more appropriate therapy. Thus, morbidity and mortality rates can be reduced significantly. The surgical reconstruction of the biliary tree and the time of onset determinate the treatment method when a biliary leakage occurs. The treatment of leaks is usually performed through endoscopic removable stenting that allows biliary drainage. Endoscopic sphincterotomy is sometime used for the removal of obstructing lithiasis or for the placement of a second stent so as to improve drainage of bile[38]. In patients in whom ERCP cannot be carried out, percutaneous transhepatic biliary drainage is usually used for diversion. In all cases in which there is a significant collection associated with a leakage, the collection must necessarily be drained in order to eliminate the risk of consequential infections and adhesions associated with the biliary fluid. In recipients with a leak shortly after transplantation or if it occurs at a hepaticojejunostomy, reintervention is generally considered[38].

US, CT, and MRC can be generally employed to identify a biliary leak[39,40]. Though imaging findings provided by these cross-sectional modalities may be suggestive of a biliary leakage in a proper clinical setting, they are frequently nonspecific (*e.g.*, fluid collection). In order to confirm the presence of an active leak, invasive procedures such as PTC or, less frequently, ERCP are generally utilized to demonstrate contrast agent extravasation from the biliary system. Nevertheless, in the current diagnostic work-up for a bile leak the first step is represented by abdominal US, and, if the findings provided by this examination are non-diagnostic, to perform conventional T2-

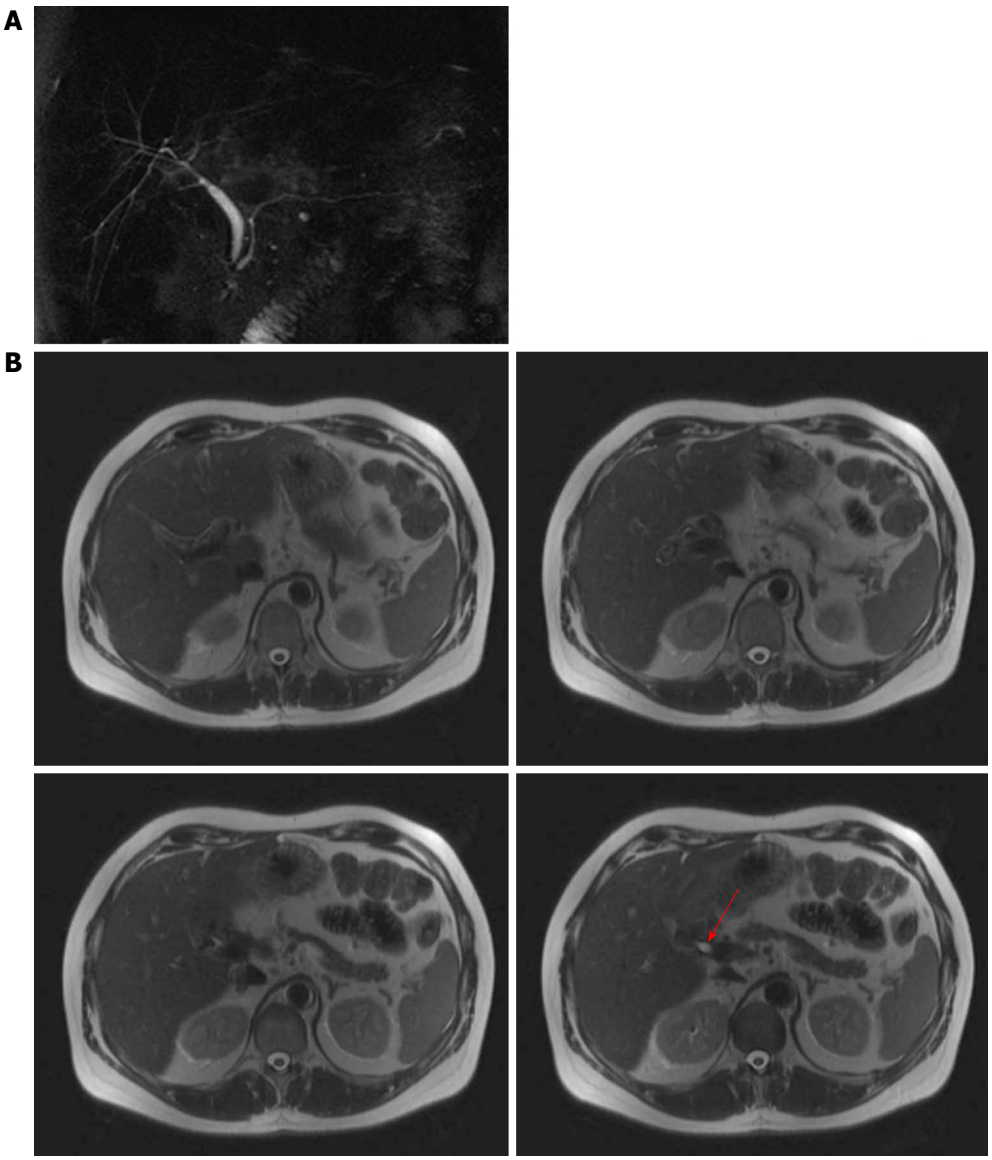

Figure 2 Duct-to-duct anastomosis. A: Single-shot thick-slab magnetic resonance cholangiogram shows a choledocho-choledocho anastomosis with a discrepancy of caliber between donor and recipient choledocho; B: Axial single-shot T2-weighted images demonstrate the anastomotic patency (red arrow).

weighted MR cholangiography. The reported accuracy of T2-weighted MRC in the detection and localization of a biliary leakage is between 70% and 74%[41].

Contrast-enhanced MRC with intravenous administration of hepato-biliary contrast agents provides functional informations as concerns as biliary excretion and may be extremely helpful in localizing the bile leak, which is not generally possible at un-enhanced T2-weighted MR cholangiography[42]. Indeed, using contrast-enhanced MRC we can demonstrate active biliary leakage by visualizing contrast media extravasation into the fluid collection and so we can also localize the anatomic site of the bile leak[43] (Figure 3).

BILIARY STRICTURES

Biliary strictures are the most frequent type of late biliary adverse events, occurring approximately 5-8 mo after OLT, and can be classified according to their location into stricture of the biliary anastomosis (AST) and non-anastomotic stricture (NAS)[44]. The incidence of the biliary strictures ranges from 5% to 34% of patients receiving liver transplant[37,45]. The prompt identification of AST and NAS is important to ensure the survival of both the organ and the patient after OLT. Moreover, the differentiation between the two types of stenosis is essential for the more appropriate therapeutic approach. Over the past few years the role of endoscopy in the management of post-OLT biliary strictures is gradually increased. Actually, the standard first-line therapy of biliary strictures is represented by endoscopic dilation with placement of single or multiple plastic stents; in most of cases and particularly in patients with anastomotic strictures this therapeutic approach avoids the need for percutaneous transhepatic therapy and surgery[46]. Non-anastomotic strictures are more difficultly treated than anastomotic

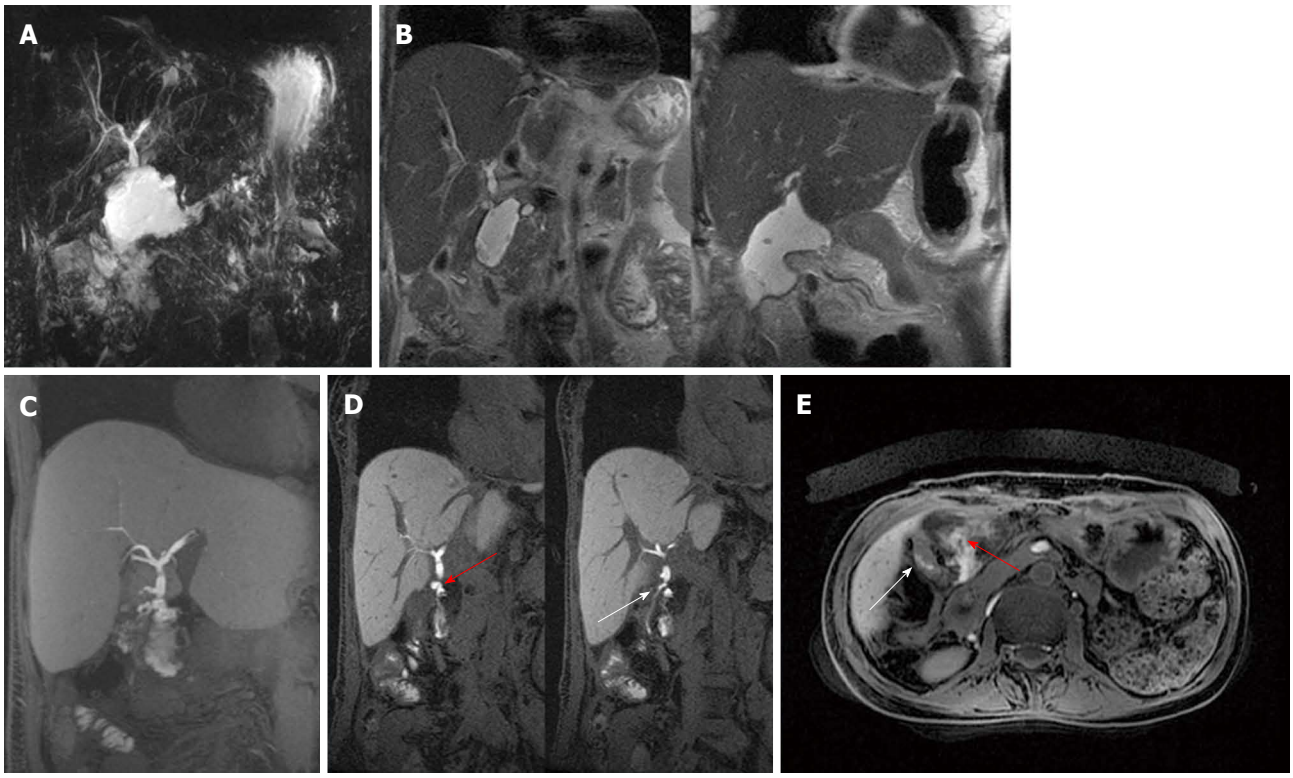

Figure 3 Anastomotic leak in a patient with hepatico-jejunostomy. A: Single-shot thick-slab magnetic resonance cholangiogram shows a fluid collection in the area of biliary-enteric anastomosis; B: Coronal T2-weighted images (at different levels) accurately depict circumscribed sub-hepatic fluid collections with thickened walls. C: Maximum intensity projection reconstruction of Gd-EOB-DTPA enhanced LAVA T1-weighted sequence well exhibits extravasation of contrast material into the peri-anastomotic space compatible with bile leak; D: Coronal Gd-EOB-DTPA enhanced LAVA T1-weighted images better identify contrast agent both extravasating from an anastomotic leak (red arrow) and filling the Roux-en-Y anastomosis (white arrow); E: On axial post-contrast LAVA image it is possible to distinguish the fluid collection (red arrow) from the jejunum (white arrow).

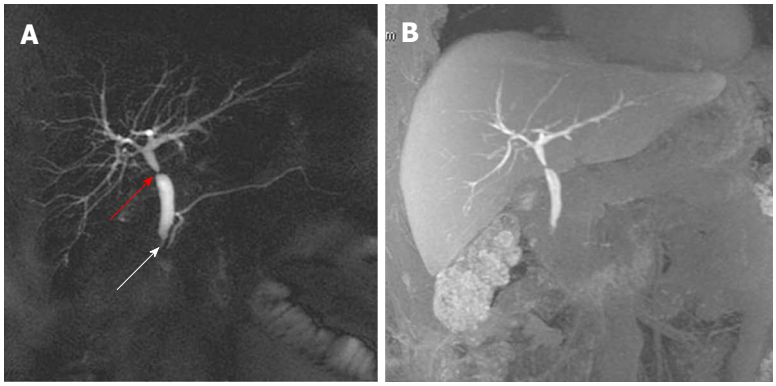

Figure 4 Anastomotic biliary stricture associated with sphincter of Oddi dysfunction. A: Single-shot thick-slab magnetic resonance cholangiogram shows stricture of both anastomotic site (red arrow) and iuxta-papillary choledocho (white arrow), with dilation of pre- and post-anastomotic biliary tract; B: Maximum intensity projection reconstruction of Gd-EOB-DTPA enhanced T1-weighted LAVA sequence demonstrates regular excretion of contrast-enhanced bile in the extra-hepatic biliary tract at 20 min, while contrast-enhanced bile excretion in the duodenum is not appreciable.

strictures, present an higher rate of episodes of cholangitis, and show a less favorable outcome in terms of graft and patient survival. A long term response to endoscopic treatment with dilatation and stent placement is reported in 50%-75% and in 70%-100% of patients with NAS and AS, respectively[46]. Percutaneous transhepatic approach and surgery including re-transplantation are currently considered for patients in whom endoscopic therapy is repeatedly failed and for those with bilio-digestive anastomosis. However, even in these latter patients, the possibilities of endoscopic treatment are expanding with the recent improvements of deep small bowel enteroscopy[46].

Anastomotic strictures at the site of biliary anastomosis can occur in both CC and CJ type of reconstruction, but they are more common after CJ than CC due to the direct bilioenteric connection[47].

In the choledochocholedochal strictures two-dimensional and 3D MR cholangiography show a circumscribed

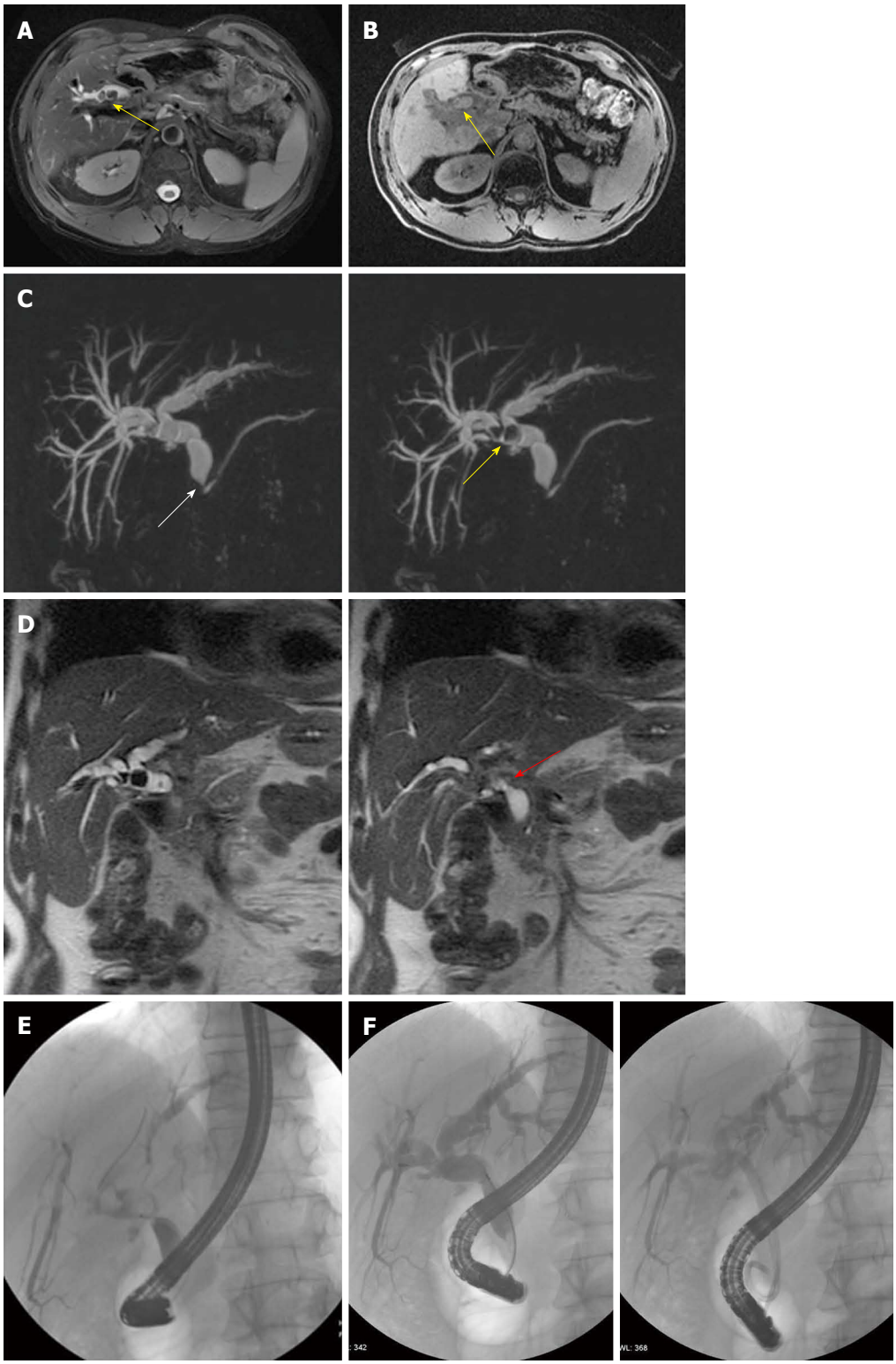

Figure 5 Anastomotic biliary stricture with lithiasis. A: Axial T2-weighted image shows dilation of the biliary system with concomitant stones (yellow arrow); B: Axial T1-weighted image confirms the presence of stones in the biliary tract (yellow arrow); C: Maximum intensity projections of 3D thin-slab fast spin-echo T2-weighted images (obtained using different thicknesses) demonstrate the dilation of the both intra- and extra-hepatic (pre- and post-anastomotic) biliary tract with a stricture of the iuxta-papillary choledocho (white arrow); the presence of two stones at the level of the hepatic bifurcation (yellow arrow) is also well appreciable; D: On coronal single-shot T2-weighted images (at different levels) is also better appreciable a stricture at the anastomotic site (red arrow); E: Endoscopic retrograde cholangiography confirms the presence of strictures and stones in the pre-anastomotic biliary tract; F: Stones were endoscopically removed and strictures were treated by stenting as shown on different projection images.

narrowing at the level of the surgical anastomosis that can be associated or not with dilatation of the pre-anastomotic biliary tract[48] (Figure 4). T1- and T2-weighted images in the axial plane show a regular thickening of

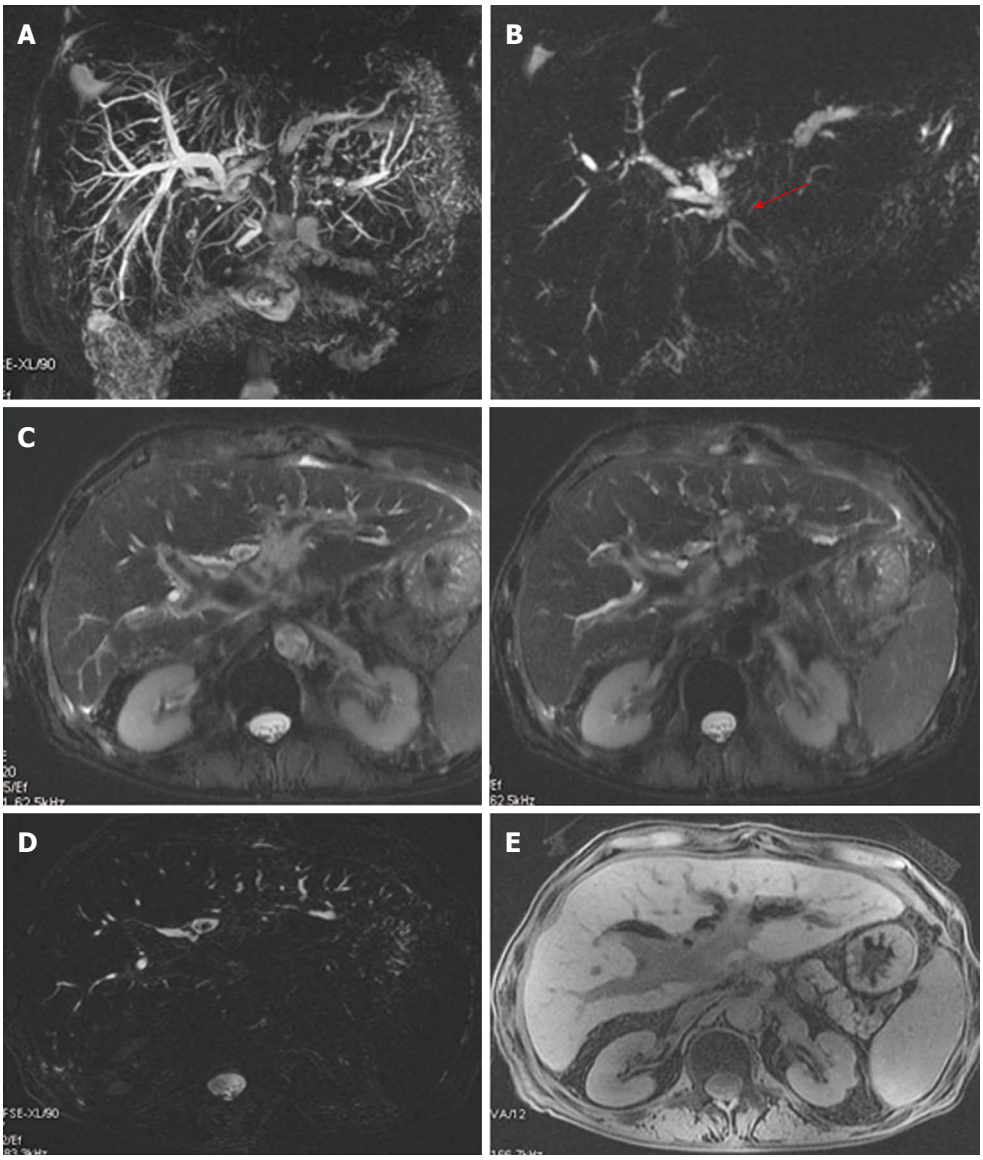

Figure 6 Anastomotic biliary stricture with lithiasis in a patient with hepatico-jejunostomy. A: Maximum intensity projection reconstruction of 3D thin-slab fast spin-echo T2-weighted images shows marked dilation of the biliary system with partial visualization of the left hepatic duct; B: Single-shot thick-slab magnetic resonance cholangiogram well depicts the stricture of the anastomotic site (red arrow); C, D: Axial single-shot T2-weighted images and axial 3D thin-slab fast spin-echo T2-weighted image demonstrate dilation of the pre-anastomotic biliary tract with the presence of pneumobilia, in particular at the level of left and common hepatic ducts with concomitant stones into the left one; E: Axial T1-weighted image better recognizes pneumobilia.

the anastomotic biliary wall with a typical ring-shaped hypointensity[48]. Besides, calculi can be appreciable in the pre-anastomotic biliary tract (Figure 5).

The surgical conformation of a biliodigestive or choledochojejunostomy make it difficult to assess with MR cholangiography. The physiological motility of the jejunal loop can cause occasional folding of the anastomotic junction with a consequent dilatation of the biliary system above the anastomosis. Besides, the bowel gas and fluid collections prevent the assessment of the anastomosis[48-50]. In a recent paper Kinner *et al*[44] concluded that post-OLT biliary stenoses can be properly identified by MRCP in recipients with CC. Nevertheless, in patients with a biliodigestive anastomosis the diagnostic performance of MRCP is reduced due to the less precise delineation of the anastomotic site (Figure 6).

Differentiation between non-obstructive *vs* obstructive dilatation of the biliary tract may be arduous on conventional T2-weighted MRC since this technique does not provide functional informations as concerns as biliary drainage[32,51]. Alternatively, high concentration of hepatobiliary contrast agents in bile ducts enables functional imaging of the biliary excretion since contrast-enhanced MRC may provide an indication of excretory function on the basis of the reference values of contrast media for biliary excretion. Furthermore, clear demonstration of the patency of the biliodigestive anastomosis can be provided by contrast agent filling of the jejunal loop on contrast-enhanced MRC (Figure 7).

Among biliary adverse events, the most troublesome are the so-called "ischemic-type biliary lesions" (ITBL), that are non-anastomotic intra- or extrahepatic stenoses

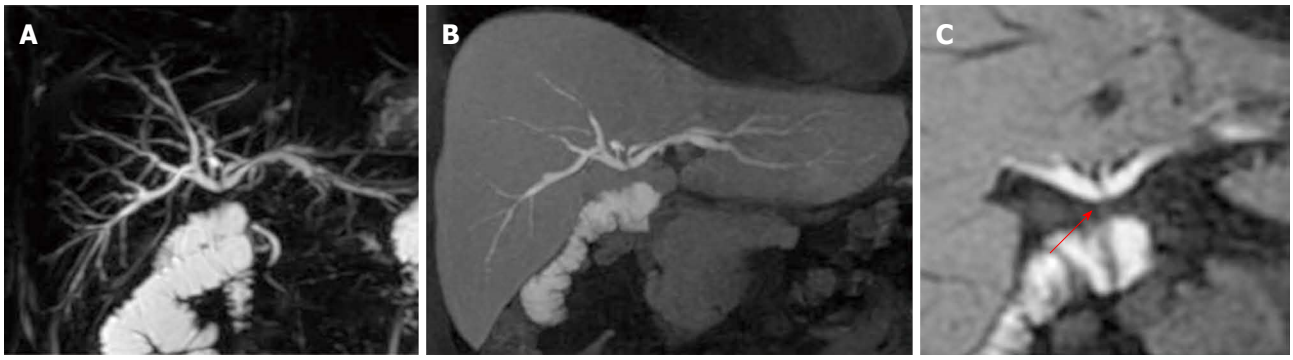

Figure 7 Patent anastomosis in a patient with hepatico-jejunostomy. A: Coronal T2-weighted magnetic resonance cholangiography reveals a moderate dilation of the intrahepatic biliary system, but does not visualize the site of the biliary-enteric anastomosis; B, C: Coronal Gd-EOB-DTPA-enhanced T1-weighted magnetic resonance cholangiogram, obtained 20 min after contrast injection, shows contrast excretion into the intrahepatic biliary system, the site of biliary-enteric anastomosis (red arrow) and anastomotic jejunal loop, demonstrating the patency of the biliary-enteric anastomosis.

and dilatations involving electively the biliary system of the transplant occurring in the absence of hepatic artery thrombosis, ABO incompatibility, or other known causes of bile duct damage. These non-anastomotic strictures have been known since the early liver transplants[52]. The appearance of these lesions suggests that microcirculatory problems related to graft preservation factors or immunogenic injury are the main pathogenic mechanisms.

Using MRC, most of ITBL show a lengthy stricture that frequently involves the right and left hepatic ducts and the hepatic bifurcation, which is a prevalent localization for ischemic injures after OLT. These stenoses more commonly start at the biliary confluence and then extend to the peripheral bile ducts, but the biliary involvement can also be intrahepatic and of various bile ducts[15]. Another characteristic feature of ITBL is represented by wall thickening of the graft common bile duct, that is generally well demonstrated on MR imaging; this findings can be sometimes associated to biliary sludge, stones or casts[15,25] (Figures 8-10). Based on MRI findings transplant surgeon can accurately assess the extension of bile ducts involvement in order to plan the more appropriate therapy and utilize ERCP only for therapeutic purposes[15].

Preliminary experiences suggest that contrast-enhanced MRC using Gd-EOB-DTPA may provide both anatomical and functional information of ITBLs in liver transplant recipients. In fact, times of contrast agent excretion seem to be in correlation with different degrees of biliary obstruction.

Several trials have evaluated the diagnostic accuracy of MRCP for the depiction of anastomotic and non-anastomotic biliary strictures. In a study by Aufort *et al*[53] twenty-seven liver transplant patients underwent MRCP using direct cholangiography as the gold standard technique. A good or excellent visualization of 80% of all biliary segments was demonstrated by MRCP. Sensitivity and specificity of MRCP for the delineation of biliary strictures were 85% and 81%, respectively. Nevertheless, these authors did not perform a separate analysis for anastomotic or non-anastomotic stenoses. In the trial by Zoepf *et al*[45] fifty liver transplant patients both with AST and NAS were examined by means of MRCP utilizing ERCP as the standard of reference. MRCP showed a sensitivity of 71% and 89% for the delineation of AST and NAS, respectively; on the other hand, the reported values of specificity were only 25% for both types of stricture. However, neither detailed description of the MRCP technique nor on the reviewers' expertise was provided in this study. Sensitivity and specificity values over 90% were reported by Boraschi *et al*[20] in a series of patients with CC undergoing MRCP. Kinner *et al*[44] evaluated the diagnostic performance of MRCP for the detection of biliary strictures after OLT according to the type of surgical reconstruction. In this trial in recipients with biliodigestive anastomosis sensitivity and specificity values of MRCP for the depiction of anastomotic and non-anastomotic biliary stenoses were 50% and 83%, 67% and 50%, respectively. Additionally, in the cohort of patients with CC the sensitivity (AST: 100%, NAS: 100%) and specificity (AST: 100%, NAS: 88%) values of MRCP were significantly higher for both types of anastomosis. Other studies have also reported an excellent accuracy of MRCP for the delineation of biliary stenoses in a inhomogeneous cohort of recipients[54,55].

At least, patients undergoing OLT for primary sclerosing cholangitis can develop multiple biliary strictures alternating with dilation of bile ducts after liver transplantation. MRCP's sensitivity is lower than that of ERCP in the identification of early alterations, but this non-invasive technique is helpful for detecting typical signs of biliary involvement in patients with a known diagnosis, in order to monitor the progress of these changes. MRCP shows beaded bile ducts or we can observe a "pruned tree" appearance of the biliary system with multiple stenoses alternating with normal or slightly dilated ducts (Figure 11).

BILIARY STONES, SLUDGE AND CASTS

Endoluminal bile duct obstruction, in the form of biliary stones, sludge and casts, can virtually occur at any time

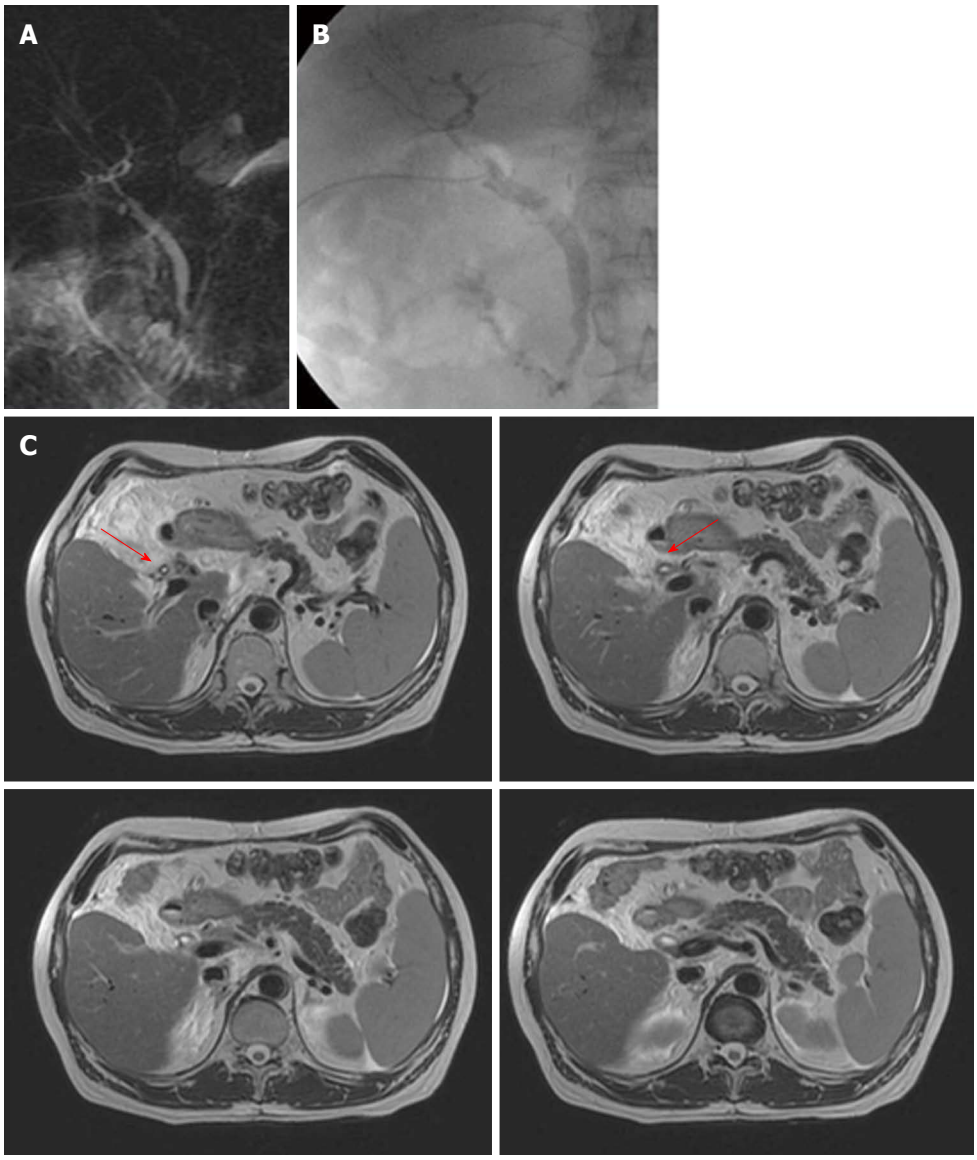

Figure 8 Non-anastomotic biliary stricture: Early ischemic type biliary lesion. A: Single-shot thick-slab magnetic resonance cholangiogram demonstrates discrepancy of caliber between donor and recipient extrahepatic biliary tract without dilation of intrahepatic biliary system; B: T-tube cholangiography confirms the presence of the mild stenosis; C: Axial T2-weighted images well exhibit the presence of circumferential wall thickening of the extrahepatic biliary tree of the graft (red arrows).

after OLT. Sludge is described as a mixture of mucous, calcium bicarbonate and crystals of cholesterol, which go on to form biliary stones if not treated. Biliary cast syndrome is characterized by an endoluminal brown, hardened material that molds to the shape of the biliary tract, leading to a "mold" or "cast" of the ductal system. The timing of onset of biliary sludge and cast syndrome is usually within the first year of transplant, while biliary stones tend to occur after. On ERCP, stones, sludge and casts are usually seen as a defect in the contrast column and described as "filling defects". Most of these latter and in particular biliary stones, are successfully treated with an endoscopic approach including sphincterotomy, lithotripsy and extraction[2].

Numerous published studies have shown that MRC is as effective as ERCP in diagnosing common bile duct stones, although the possibilities of MRI in identifying calculi of a few millimeters in size are still to be fully proven[56]. Endoscopic ultrasound (EUS) is able to provide images of high resolution since the endoscopic probe is in close proximity to the internal structures. The spatial resolution of EUS is higher than that of MRCP (0.1 mm *vs* 1.5 mm) and consequently this technique is extremely reliable for the detection of small calculi also for the advantage of providing dynamic images compared to MRCP[57]. Nevertheless, since EUS is an invasive procedure and requires sedation of the patient, the need to perform it in low risk subjects should be carefully evaluated. In fact, even a merely diagnostic EUS may cause complications such gastro-intestinal bleeding and bowel perforation[57]. In a recent paper Verma *et al*[57] compared the diagnostic performance of EUS and MRCP for the identification of common bile duct stones when using the data from published prospective comparative stud-

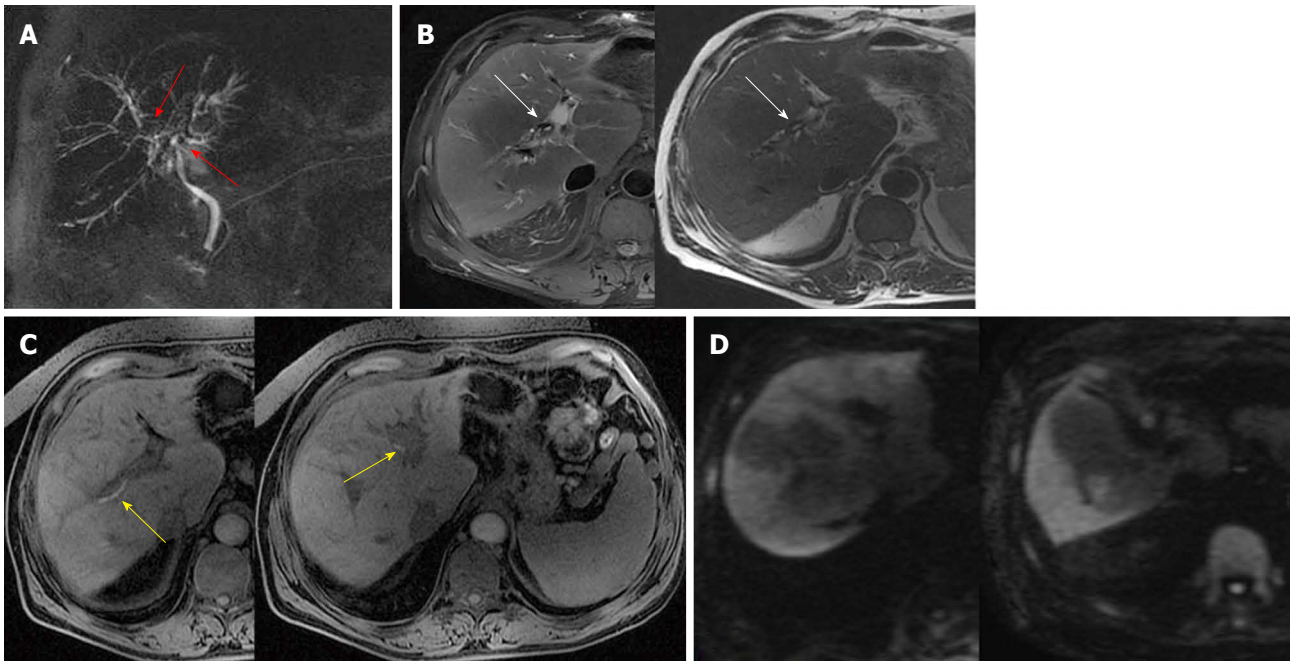

Figure 9 Non-anastomotic biliary stricture: Advanced ischemic type biliary lesion. A: Single-shot thick-slab magnetic resonance cholangiogram demonstrates a stenosis of the hepatic bifurcation and hepatic ducts (red arrows) with an irregular dilation of the intrahepatic biliary system; B, C: Axial T2- and axial T1-weighted images well exhibit the presence of circumferential wall thickening (white arrows) at the level of hepatic bifurcation and endoluminal casts (yellow arrows); D: On diffusion-weighted imaging the liver parenchyma appears inhomogeneous with multiples areas of persistent high signal intensity in highest *b*-value acquisitions.

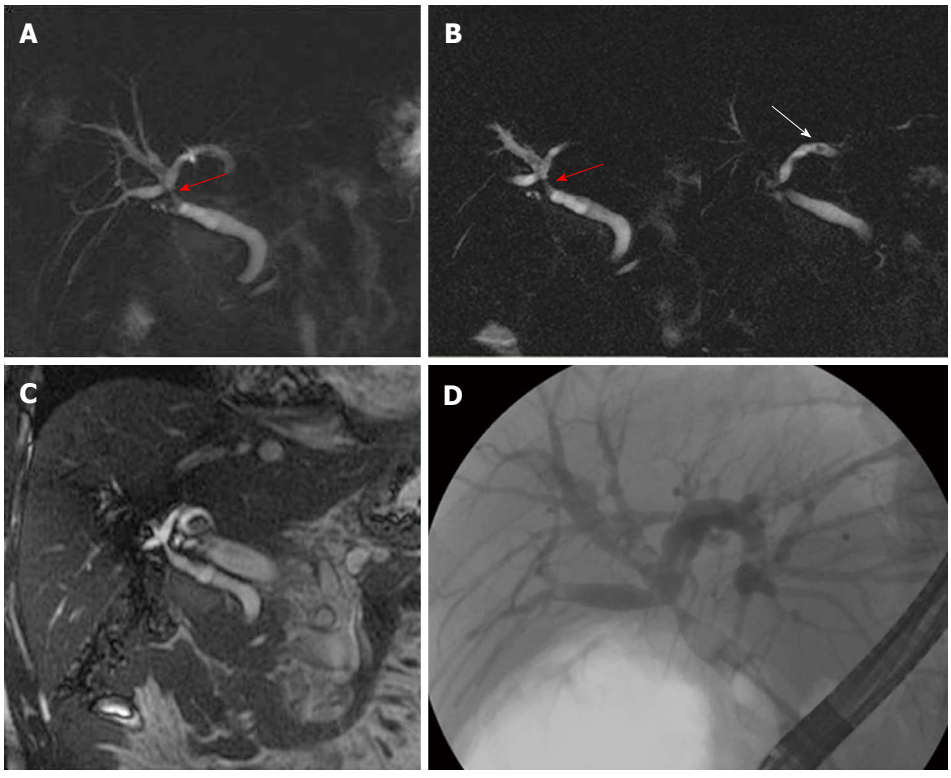

Figure 10 Non-anastomotic biliary stricture: Classic signs of ischemic type biliary lesion. A, B: Single-shot thick-slab magnetic resonance cholangiopancreatography show the stricture at the level of hepatic bifurcation (red arrow) and the presence of biliary sludge/stones (white arrow) in the dilated intrahepatic biliary system; C: On coronal T2-weighted image the wall thickening of the extrahepatic biliary tree of the graft is also well appreciable; D: Endoscopic retrograde cholangiography exhibits the optimal correlation of these features.

ies. On the whole, no significant difference was found between these two tests for the diagnosis of choledocholithiasis, though both techniques showed high diagnostic accuracy. The authors concluded that factors such as pa-

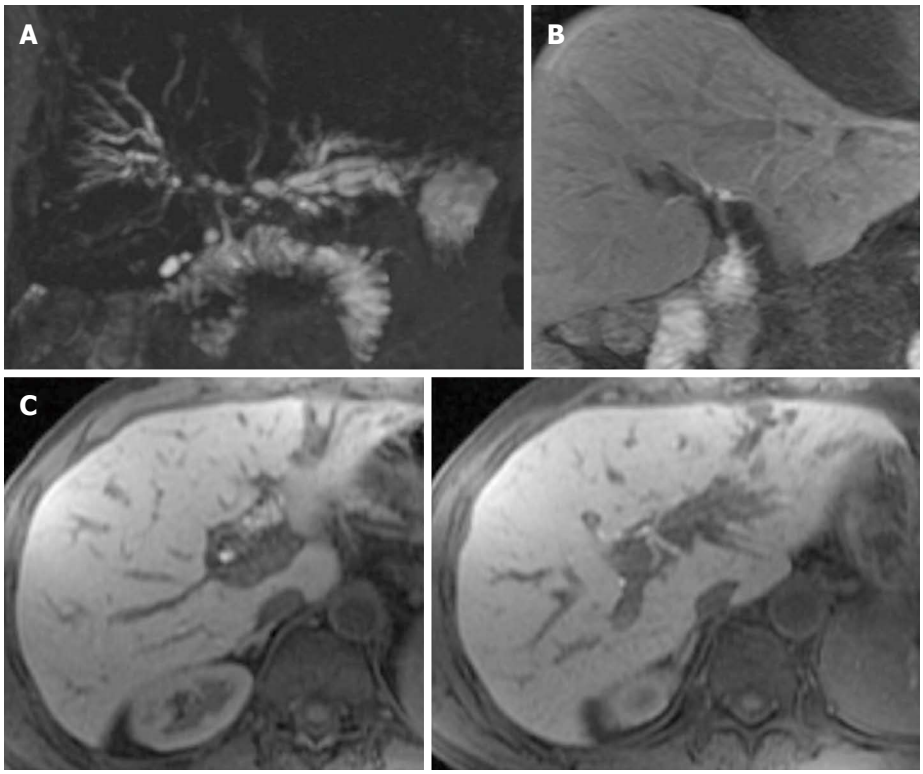

Figure 11 Recurrence of primary sclerosing cholangitis in a transplant patient with hepatico-jejunostomy. A: Maximum intensity projection (MIP) reconstruction of 3D thin-slab fast spin-echo T2-weighted images shows multifocal stenosis with intervening saccular dilation affecting the intrahepatic biliary system; B, C: On MIP reconstruction and axial Gd-EOB-DTPA enhanced LAVA T1-weighted sequences the intrahepatic biliary system is not appreciable, however the irregularities of the extrahepatic biliary tract are more accurately depicted.

tient suitability, expertise and costs should be considered when deciding between EUS and MRCP[57].

On conventional T2-weighted MR cholangiography the presence of pneumobilia is an element that can compromise the correct diagnosis of lithiasis. The differential diagnosis between stones and pneumobilia is usually performed on axial T2-weighted sequences. Calculi are generally identified as endoluminal areas of signal void surrounded by high intensity of bile in the dependent portion of the duct (Figure 5), whereas pneumobilia is typically characterized by low signal intensity in the non-dependent portion of the bile duct[19] (Figure 6). Besides, on conventional T2-weighted MRC flow artifacts are sometimes observed in the central portion of choledochal duct as thin area of low signal intensity[58]. These flow artifacts are not commonly recognized on contrast-enhanced T1-weighted MR cholangiography, that may be helpful in providing an increased diagnostic confidence in the differential diagnosis between stones and pneumobilia. Furthermore, Kinner *et al*[59] showed that adding non-enhanced T1-weighted sequences to conventional T2-weighted MRCP the diagnostic performance of MRI for the diagnosis of biliary cast syndrome after OLT is significantly improved since biliary cast is hyperintense on T1-weighted images (Figure 9).

In liver transplant patients biliary obstruction caused by lithiasis is usually associated with an anastomotic stricture. Moreover, a biliodigestive by-pass (even if patent) is a factor encouraging the development of biliary stones[19]. Clinically these patients can present typical signs of cholangitis, represented by abdominal pain, fever, and jaundice, the classic Charcot triad. If an obstruction of the biliary system is not promptly recognized, patients may develop ascending cholangitis, showing multiple intrahepatic biliary strictures that mimic the features of primary sclerosing cholangitis[19].

SPHINCTER OF ODDI DYSFUNCTION

Another common occurrence after OLT is represented by sphincter of Oddi dysfunction (SOD) that is reported to be up to 7% in liver transplant recipients. The pathogenesis of SOD is attributed to the denervation of the sphincter during liver transplantation. This leads to an increase in basal pressure, thus causing increased pressures in the choledochal duct and, as a consequence, a mild increase in the size of donor and recipient common bile ducts[60]. There have been virtually no clinical trials that demonstrate the best treatment option for SOD. In recent years, endoscopic therapy with sphincterotomy with or without stenting has been the most acceptable treatment option for SOD in liver transplant recipients.

On MRCP we can observe a significant dilatation of both recipient and donor bile duct in the absence of cholangiographic evidence of obstruction; a protrusion of the enlarged ampullary region into the duodenal lumen is sometimes associated (Figure 4). In these cases, SOD is suspected and contrast-enhanced MR imaging

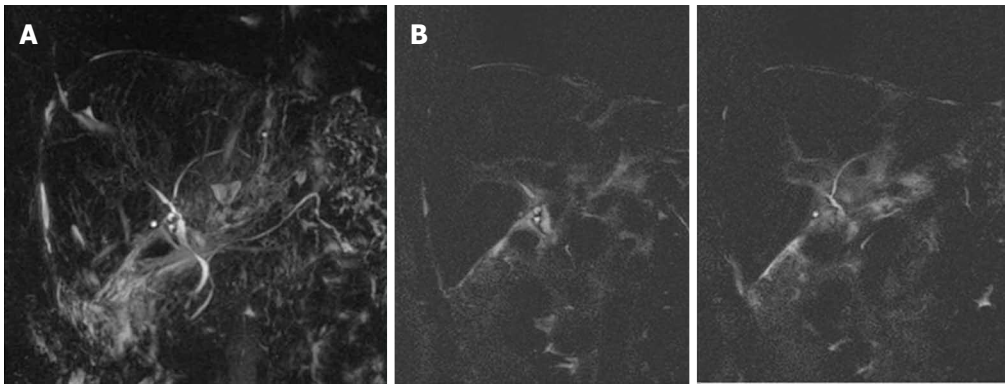

Figure 12 Vanishing bile duct syndrome. A: Maximum intensity projection reconstruction of 3D thin-slab fast spin-echo T2-weighted images shows only extrahepatic pre- and post-anastomotic biliary tract; the entire intrahepatic biliary system is missed; B: These findings are better appreciable on single-shot thin-slab magnetic resonance cholangiograms.

can be added to T2-weighted MR cholangiography in order to obtain functional information on the degree of biliary obstruction and increase the diagnostic accuracy of MR imaging, particularly in patients with biochemical abnormalities that could be treated endoscopically with or without stenting.

VANISHING BILE DUCT SYNDROME

Vanishing bile duct syndrome is characterized by progressive loss of small intrahepatic ducts caused by a variety of different diseases leading to chronic cholestasis, cirrhosis, and premature death from liver failure[61]. In post liver transplantation patients acute and chronic rejection and ischemia are the most common causes. The diagnosis is usually established by liver biopsy in the appropriate clinical setting and treatment depends mainly on the underlying etiology of the disease. On MRCP images this disease entity, also referred as ductopenia, can be suspected when we observe a paucity of small intrahepatic bile ducts (Figure 12).

CONCLUSION

Biliary adverse events following OLT are relatively common and continue to be important causes of morbidity, mortality, and transplant dysfunction or failure.

MR imaging with MRCP sequences is crucial in assessing abnormalities in the biliary system after liver surgery, including liver transplant. MR cholangiopancreatography is a safe, rapid, non-invasive, and effective diagnostic modality for the evaluation of biliary adverse events after OLT, since it plays an increasingly important role in diagnosis and management of these events. It can provide a non-invasive method of imaging surgical reconstruction of the biliary anastomoses as well as adverse events including anastomotic and non-anastomotic strictures, biliary lithiasis and sphincter of Oddi dysfunction in liver transplant recipients. Nevertheless, conventional T2-weighted MR cholangiography can be implemented with T1-weighted contrast-enhanced MR cholangiography using hepatobiliary contrast agents (in particular using Gd-EOB-DTPA) in order to improve the diagnostic accuracy in the adverse events' detection such as biliary leakage and strictures, especially in selected patients with biliary-enteric anastomosis.

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P- Reviewer: Abbasoglu O, Ogura T, Sira MM
S- Editor: Wen LL **L- Editor**: A **E- Editor**: Zhang DN

World Journal of Gastroenterology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i32.11095

World J Gastroenterol 2014 August 28; 20(32): 11095-11115
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (7): Liver transplant

Post-liver transplant hepatitis C virus recurrence: An unresolved thorny problem

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Received: December 27, 2013 Revised: February 15, 2014
Accepted: May 29, 2014
Published online: August 28, 2014

Abstract

Hepatitis C virus (HCV)-related cirrhosis represents the leading cause of liver transplantation in developed, Western and Eastern countries. Unfortunately, liver transplantation does not cure recipient HCV infection: reinfection universally occurs and disease progression is faster after liver transplant. In this review we focus on what happens throughout the peri-transplant phase and in the first 6-12 mo after transplantation: during this crucial period a completely new balance between HCV, liver graft, the recipient's immune response and anti-rejection therapy is achieved that will deeply affect subsequent outcomes. Nearly all patients show an early graft reinfection, with HCV viremia reaching and exceeding pre-transplant levels; in this setting, histological assessment is essential to differentiate recurrent hepatitis C from acute or chronic rejection; however, differentiating the two patterns remains difficult. The host immune response (mainly cellular mediated) appears to be crucial both in the control of HCV infection and in the genesis of rejection, and it is also strongly influenced by immunosuppressive treatment. At present no clear immunosuppressive strategy could be strongly recommended in HCV-positive recipients to prevent HCV recurrence, even immunotherapy appears to be ineffective. Nonetheless it seems reasonable that episodes of rejection and over-immunosuppression are more likely to enhance the risk of HCV recurrence through immunological mechanisms. Both complete prevention of rejection and optimization of immunosuppression should represent the main goals towards reducing the rate of graft HCV reinfection. In conclusion, post-transplant HCV recurrence remains an unresolved, thorny problem because many factors remain obscure and need to be better determined.

Key words: Hepatitis C virus; Liver transplantation; Hepatitis C antigens; Graft rejection; Immunosuppression

Core tip: Hepatitis C virus (HCV) graft reinfection universally occurs post-liver transplantation and disease progression is accelerated. Differentiating recurrent hepatitis from rejection is essential in this setting; however, differentiation of the two pathological patterns remains difficult. The host immune response appears to be crucial both in the control of HCV infection and in the genesis of rejection: complete prevention of rejection and optimization of immunosuppression should represent the main goals. A proper graft allocation seems to be crucial to realize an ideal donor-to-recipient matching; however, many factors remain obscure.

Grassi A, Ballardini G. Post-liver transplant hepatitis C virus recurrence: An unresolved thorny problem. *World J Gastroenterol* 2014; 20(32): 11095-11115 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11095.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11095

INTRODUCTION

Since the liver transplant (LT) was approved as a life-saving intervention for end-stage liver disease in the 1980s, decompensated cirrhosis from hepatitis C virus (HCV) has become, and will remain, the leading cause of LT in developed, Western and Eastern countries[1-3]. Unfortunately, LT does not cure the recipient's HCV infection. Reinfection occurs universally and disease progression is accelerated compared with the non-transplanted population. Histologically proven hepatitis C-related cirrhosis can be documented within a mean of five years after transplantation and, from that point on, the first episode of decompensation may occur in less than one year. Graft failure and loss are the unavoidable result for about 30%-35% of patients, resulting in poor outcomes for HCV-infected recipients compared with those who are HCV-negative[4-6].

In this review we discuss the current understanding of graft HCV reinfection in the LT recipient, with a focus on the peri-transplant phase and the first 6-12 mo after transplantation: during this crucial period, a completely new balance between HCV, liver graft, recipient immune response and anti rejection therapy is achieved, which will deeply affect subsequent outcomes. We have divided this article into five sections, focusing on the following pivotal topics: (1) early dynamics of graft HCV reinfection post LT; (2) differentiating acute cellular rejection (ACR) from early recurrent hepatitis C (RHC); (3) the role of genetic and host immune response; (4) immunosuppressive treatment and immunotherapy; and (5) graft-related factors.

EARLY DYNAMICS OF GRAFT HCV REINFECTION POST LT

Serum HCV RNA

Reinfection (measured by detectable serum HCV RNA) is the universal outcome after LT for HCV-related liver disease[7,8]. Serum viral load reflects a complex interaction between viral production by infected cells and clearance by the host immune system. After LT, the relative contribution of each of these factors likely differs at different sampling times.

Serum HCV RNA decreases rapidly during, and immediately after, the removal of the infected liver and the implantation of the new, uninfected graft. This is followed by a steady increase in viral concentrations within days. Garcia-Retortillo *et al*[9] showed that serum viral load rapidly decreases with reperfusion of the allograft, presumably as the liver removes virus from the circulation and the intrahepatic viral amount increases (intrahepatic viral load was not determined in this study); serum viral load reached a nadir 8-24 h after reperfusion, likely representing saturation of cell surface receptors for HCV in the allograft. The subsequent increase in the serum viral load should represent established infection and production of new virus by the infected allograft. During the first week following transplantation, viral kinetics appear highly variable between individuals and may be related to an attenuated immunological response of the recipient[9]. Once the new liver becomes infected, hepatic viral replication resumes, with serum HCV RNA reaching and exceeding pre-LT levels[4,10-17]. The rapid increase of HCV viral loads after LT proves the high capacity of HCV to adapt to a new environment. In particular, viral escape from a dominant immune response early after LT could play a central role in viral persistence by enhancing viral survival when it is most susceptible to immune selection, as in case of massive infection of the graft[18].

Not all allografts are equally efficient hosts for viral replication: Negro *et al*[11] showed that rates of viral replication in allografts (determined by anti-genomic strand-specific real time-polymerase chain reaction) appear to differ between patients and seem to be not related to immunosuppression. Limitations of this study were its semi-quantitative nature and the lack of clear defined protocol biopsy time-points[11].

The impact of post-LT serum viral load on clinical prognosis remains unclear: some authors argued that viral load does not seem to be correlated with worse outcomes in the post-transplant setting[19,20]; however, more recently, others have shown that high levels of replication at this time are correlated with the development of additional fibrosis in the allograft at one year post-transplant[21] and are associated with increased patient mortality and liver-related mortality[22].

These results are likely confounded by many factors: blood loss, transfusions and ongoing resuscitation during surgery; furthermore, secondary sites of viral infection may also contribute to variability in amount of virus available to infect the liver[23].

Powers *et al*[24] carefully evaluated six HCV-positive patients, collecting very frequent blood samples during and soon after the anhepatic phase in the course of LT. During the pre-anhepatic and anhepatic phases, HCV RNA levels dropped with an average half-life of 0.8 h and begin to rise (doubling-time 2.0 d) only 15 h after the anhepatic phase. Based on the decline in viral load over the first 24 h of the post-anhepatic phase, the authors estimated that a non-hepatic source might contribute up to 4% of total viral production, confirming data reported by Dahari *et al*[25] who evaluated that this extra-hepatic compartment is responsible for about 3.1% of virus in circulation.

HCV quasispecies and subpopulations

Other studies of viral replication have also examined HCV quasispecies evolution during first few days post LT.

Feliu *et al*[26] showed a reduced viral complexity with respect to pre-transplant levels, suggesting a "bottleneck" effect, which arose soon after LT such that only one part of the pre-transplant variants reinfects the graft.

In contrast to the "bottleneck" scenario, Gray *et al*[27] revealed that multiple HCV lineages are transmitted at

the time of LT, without a major decrease in viral genetic diversity. Although only some of the pre-transplant lineages were identified within the first 4 mo post-transplant, lineages are undoubtedly present because their ancestors were sampled at later time points. It should be underlined, (as correctly reported by the authors themselves) that all virus populations in that study were obtained from serum and, although such viruses are often assumed to represent the viral population in the liver, they may also contain variants from non-hepatic sites.

Other authors demonstrated that allografts remove from the circulation, and are infected by, certain HCV subpopulations over others in the immediate post-operative period. This selection for a fraction of HVR1 (the second envelope protein at hypervariable region 1) variants by allografts suggests that this area of the viral envelope contributes significantly to viral-allograft interaction. Additionally, after transplant, allografts express variable amounts of CD81, a multifunctional protein that has been demonstrated to act as a cell surface receptor for HCV and may interact directly with HVR1[19,28].

Other authors focused their attention on SR-BI, an 82-kDa glycoprotein highly expressed in the liver. SR-BI binds a variety of lipoproteins (HDL and LDL) and is involved in bidirectional cholesterol transport across the cell membrane[29]. It has been suggested that the interplay between lipoproteins, SR-BI, and HCV envelope glycoproteins is required for HCV entrance into liver cells[30,31]. In the setting of LT, Meuleman *et al*[32] demonstrated that a human monoclonal antibody targeting SR-BI efficiently precluded HCV infection and viral spread after LT, both *in vitro* and *in vivo*.

In a small, but very precise, analysis of six patients infected by HCV genotype 1b who underwent LT, HCV variants reinfecting the liver graft were characterized by efficient entry and poor neutralization by antibodies present in pre-transplant serum. Conversely, pre-transplant subvariants not detected soon after LT were characterized by less effective hepatocyte entry[33].

Nevertheless, the clinical significance of quasispecies evolution with established infection remains controversial. Sullivan *et al*[34] found that higher levels of diversity correlate with less severe recurrence (presumably because of a stronger immune response to the virus), whereas Pessoa *et al*[35] showed that immunosuppressed transplanted patients have greater quasispecies diversity than immunocompetent non-transplanted patients.

Hughes *et al*[28] demonstrated that only a portion of the complex population of quasispecies present in patient serum before reperfusion of allografts goes on to infect the liver, and that this quasispecies selection begins immediately upon reperfusion. It seems possible that persistence of a predominant variant from pre-transplant serum to post-perfusion liver would result in a greater magnitude of liver infection. This appears to be in agreement with previously reported data[36,37], where persistence of a predominant serum variant from pre- to post-transplant serum was associated with RHC, whereas failure of predominant variants to persist post-transplant was associated with no early recurrence. On the contrary, Pessoa *et al*[35] found that in the subset of patients with fibrosing cholestatic hepatitis (a severe form of HCV recurrence associated with early graft failure and death), divergence of quasispecies is enhanced, resulting in the emergence of many new variants. In a peculiar model of superinfection (HCV-infected liver into an HCV-positive recipient), Vargas *et al*[38] demonstrated that superinfection of the liver by the donor strain is associated with significantly milder disease than when the recipient strain becomes dominant. In addition, genotype 1 consistently predominates over non-1 genotypes in recipients of infected grafts, suggesting replicative differences among viral strains.

HCV genotype and influence of co-infections

The influence of HCV genotypes on RHC is still controversial: some studies demonstrated that the severity of recurrence and the levels of HCV viral replication after LT are higher in patients with genotype 1b infection than in those with other genotypes[4,39-41]. By contrast, Gayowski *et al*[42] reported that the rate and the severity of RHC does not differ among genotypes, suggesting that HCV genotype might not be a significant factor influencing post-LT HCV hepatitis.

Some authors have proposed that cytomegalovirus (CMV) and human herpes virus-6 may have an immunomodulatory effect in transplanted recipients, and might play a role in promoting HCV replication[43,44]. Bosch *et al*[45] considered 347 LT recipients (donor or recipient CMV seropositive) transplanted for HCV related liver disease retrospectively to evaluate the associations of CMV infection and disease with RHC after LT. They demonstrated that CMV infection was associated with increased risk of fibrosis stage $\geqslant 2$ and grade of inflammation $\geqslant 2$. By contrast, Nebbia *et al*[46] reported that short term CMV viremia did not increase the replication of HCV after LT.

In light of these contrasting data, the clinical significance of the degree of, and variations in, early quasispecies complexity, and the influence of HCV genotype or other viruses on HCV replication post-LT, remain mostly unclear.

Evaluation of intrahepatic HCV

The measurement of the amount of HCV in the serum and its dynamic evolution may be less relevant than the amount of virus in the liver. Intrahepatic viral load rather than freely circulating virus likely causes liver injury; therefore, liver viral load may better reflect the magnitude of infection than serum viral load. While Terrault *et al*[47] found that serum and liver viral loads differed widely (ratio of liver/serum viral load ranged from 17 to 286), Sreekumar *et al*[21] demonstrated that serum and liver viral loads were significantly correlated ($r = 0.77$-0.93, $P < 0.01$), though intrahepatic levels were always higher (on average by 79-fold). It should be noted

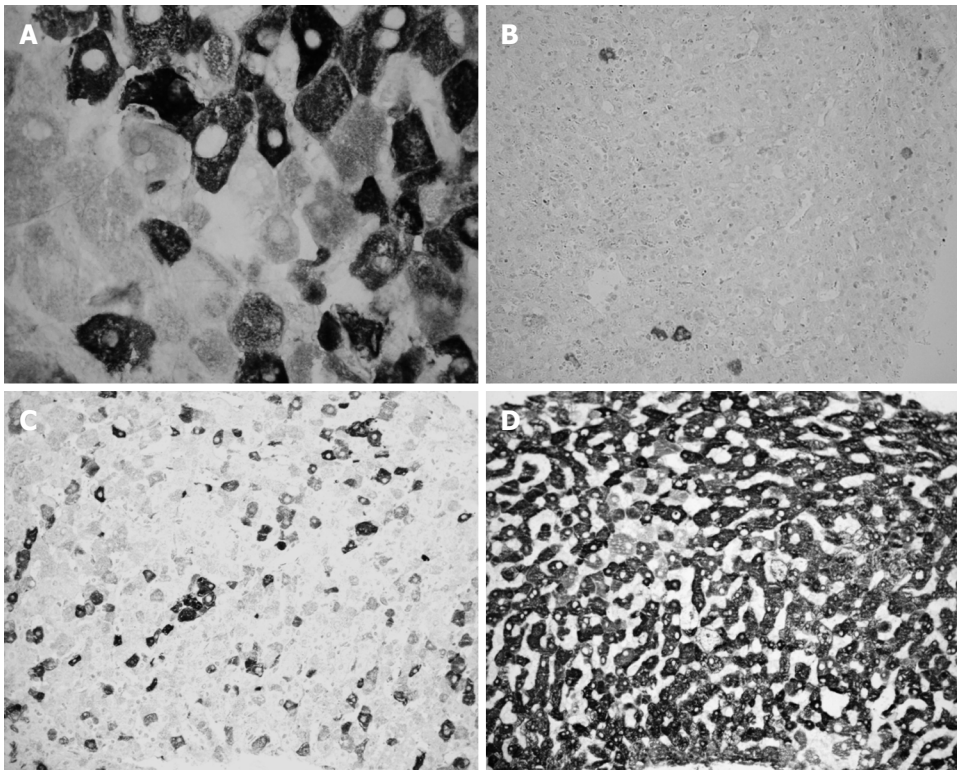

Figure 1 Immunohistochemistry of lobular areas from different liver biopsies stained for hepatitis C virus-antigens. Cytoplasmic positivity of hepatocytes with different intensities of staining. A: Negative and strongly positive hepatocytes in the same areas. Original magnification 120 ×; B: Few positive hepatocytes. Original magnification 20 ×; C: About 20% positive hepatocytes with different intensity of staining. Original magnification 20 ×; D: Widespread positivity, with prevalent strong intensity of staining. Original magnification 20 ×.

that the authors obtained different results despite using the same technique: these differences may reflect the narrow dynamic range of detection for their assays (early generation branched DNA), which allows discrimination of a 3-log range of concentrations only.

Fewer data are available concerning the dynamics of HCV reinfection within the graft and the liver expression of HCV antigens.

Liver HCV antigens expression is detected very early post-LT: 25% of liver specimens obtained within 10 d post LT show HCV antigens expression. This percentage rises to 66% and 90% when liver samples are collected between 11 and 20 or 21-60 d post-LT, respectively[48]. A subsequent paper demonstrated that the expression of liver HCV antigens is common until six months post-LT (92% of frozen liver specimens), while it declines after six months post-LT (74% of frozen liver specimens)[49], (Figure 1). Accordingly, Mensa *et al*[50] demonstrated on formalin fixed-paraffin embedded liver specimens that HCV core protein expression is present in 75% and 33% of acute phase and follow-up biopsies post-LT, respectively.

DIFFERENTIATING ACR FROM EARLY RHC

Differentiating between ACR and early RHC after LT is a challenging histological and clinical problem in the management of patients transplanted for HCV-related cirrhosis. In fact, both pathological conditions are associated with lymphocytic infiltration and variable degrees of bile duct injury in the portal tracts, as well as the presence of centrilobular necrosis. Clinically, increased aminotransferase and bilirubin levels characterize both diseases, whereas HCV blood viral load is of little help; moreover, both diseases may coexist.

The differentiation of RHC from ACR is crucial for appropriate treatment. Incorrect diagnosis may be detrimental, as failure to increase immunosuppression in patients with ACR may lead to acceleration of rejection. More importantly, increasing immunosuppression to treat presumed rejection may worsen RHC and lead to a faster progression to fibrosis and cirrhosis of the graft[13,51-54]. There is limited information on the reliability of histopathological assessment for the differentiation of RHC from ACR post-LT. One study in a small group of patients demonstrated relatively low interobserver and intraobserver agreement rates between two pathologists in early post-transplant liver biopsies[55]. More recently, Regev *et al*[56] evaluated interobserver agreement between five pathologists on the histopathological diagnosis in 102 liver biopsy specimens from post-LT HCV-positive patients. They revealed a slight agreement (*K* score = 0.12) on the histopathological diagnosis. All five pathologists agreed on the diagnosis of RHC in only

five patients (5%) and on the diagnosis of ACR in only two patients (2%). Moreover, the intraobserver agreement also showed low reliability. Distinguishing RHC from ACR may be difficult, especially in the early stages of RHC, as both RHC and ACR may be associated with lymphocytic infiltration of the portal tracts and variable degree of bile duct injury with occasional lymphocytic aggregates. Thus, histology should be used very cautiously for differentiating RHC from ACR post-LT.

To improve the possibility of discriminating ACR from RHC we evaluated the percentage of HCV-infected hepatocytes using an immunohistochemical technique based on FITC-conjugated human polyclonal anti-HCV immunoglobulins in 55 frozen biopsy specimens from post-LT HCV recipients. The number of HCV-infected hepatocytes was never less than 40% in acute hepatitis specimens and never greater than 30% in the other cases; therefore, the detection of liver HCV antigens might be useful, combined with conventional histological evaluation, to make a diagnosis of RHC[48]. In a wider series (215 liver specimens) using the same technique, we found that in 15 out of 118 (13%) specimens obtained within six months post-LT, a final diagnosis of recurrent hepatitis occurred during the follow-up, despite previous inconclusive or discordant histological diagnosis. In all these patients, many infected hepatocytes were detected. Moreover, the presence of more than 30% HCV-infected hepatocytes confirmed the presence of RHC and "absolutely" excluded the presence of significant rejection[49]. These data were confirmed by Sadamori *et al*[57] using a similar immunohistochemical technique with a monoclonal antibody against HCV-envelope 2 in a series of 84 liver biopsies.

Other authors considered 65 liver specimens comparing tissue HCV quantification and HCV immunohistochemistry (IHC) to histology. They demonstrated that HCV RNA, HCV IHC, and Councilman body/portal tract ratio are the only variables able to discriminate ACR. They therefore proposed to routinely perform at least HCV RNA tissue quantification, in addition to histology, in all initial biopsies performed after LT in HCV-positive patients[58]. The same authors described stratification of the risk of RHC post-LT using tissue and serum HCV RNA quantification. In a series of 83 post-LT liver specimens they reported that when tissue HCV RNA is $\leqslant$ 1.5 IU/ng with any serum HCV RNA, the recurrent hepatitis rate was 61%. By contrast, when tissue HCV RNA was > 1.5 IU/ng the recurrent hepatitis rate was 91%, if serum HCV RNA < 40×10^6 copies/mL, and 100%, if serum HCV RNA > 40×10^6 copies/mL[59].

Ciccorossi *et al*[60] focused their attention on IgM anti-HCV in a series of 98 consecutive HCV-positive LT patients. They found that the serum IgM anti-HCV titer increased in 82% of cases with RHC, while remaining unchanged in all rejection cases. Moreover, the IgM anti-HCV titer increased in 10 of 11 histologically doubtful cases that were diagnosed as hepatitis at the subsequent liver biopsy. Thus, they proposed the quantitative monitoring of IgM anti-HCV as an additional diagnostic tool for distinguishing RHC from graft rejection.

Other authors reported that C4d (a marker of the activated complement cascade) is detectable in hepatic specimens in acute rejection after LT. They analyzed retrospectively 97 paraffin embedded specimens by immunohistochemistry, and demonstrated that 67.7% of patients with ACR showed C4d-positive staining in liver biopsy compared with 11.8% of patients with hepatitis C reinfection. The hypothesis is that humoral components, represented by C4d deposition, might play a role in ACR after LT and might be helpful to distinguish between acute rejection and HCV reinfection[61]. Nevertheless, the same authors were not able to confirm these results using ELISA measurement of C4d concentration in a prospective series of cryo-preserved liver biopsies from post-LT patients[62].

Transcriptional analysis has also been applied to explore potential pathways defining the presence of ACR in the setting of recurrent HCV infection after LT. Microarray analysis has identified differentially expressed genes associated with a variety of pathways, including apoptosis, as potentially targeting the presence of ACR in this setting[63].

Joshi *et al*[64] analyzed liver micro RNA (miRNA) expression in a carefully matched series of patients who had previously undergone LT for HCV-related liver disease, comparing those with slow *vs* rapid fibrosis progression, individuals with ACR, and control subjects without viral hepatitis. A clear segregation of miRNA expression patterns was seen for all four groups. A pathway analysis that compared subjects with slow fibrosis and subjects with rapid fibrosis revealed differences in miRNA expressions influencing antifibrotic, antiangiogenic, anti-inflammatory and antiapoptotic pathways. These results identified a number of potential pathways for further exploration with respect to the pathogenesis of RHC after LT, as well as potential biomarkers useful to detect rapid fibrosis progression and ACR in this setting. The main bias of this otherwise intriguing paper is the different timing of sampling biopsies. Patients with slow or rapid fibrosis progression had protocol liver biopsy one year after transplant, when fibrosis was already well established. By contrast, liver biopsies from patients with histologically diagnosed ACR were obtained at the time of suspected rejection. Thus it is not clear whether the observed changes in mRNA expression predict the development of a specific injury phenotype, or simply are the result of established differing patterns of injury within the allograft[64].

Recently Cabrera *et al*[65] proposed a blood test to discriminate ACR from RHC post LT, focusing on what happens in the blood rather than in the liver. Using the ImmuKnow assay, which measures the amount of ATP produced by CD4 lymphocytes after stimulation, they studied 42 transplanted patients. Patients with ACR presented a significantly stronger immune response

than those with active RHC, while patients with mixed features of ACR and mild RHC showed an intermediate immune response[65]. The main advantage of this assay is the rapid assessment of nonspecific CD4 effector T-cells. Responses within 24 h offer real-time results on the status of the cell-mediated function, whereas the traditional functional immunological assays need long incubation periods. Obviously, as correctly reported by the authors, these data need to be confirmed in a larger population of transplanted patients.

ROLE OF GENETICS AND THE HOST IMMUNE RESPONSE

Human leukocyte antigens system

The major histocompatibility complex (MHC) in LT plays a less important role than in other solid organs because the liver is more tolerogenic and most allograft losses are caused by recurrence of the primary disease rather than by rejection. Even if human leukocyte antigen (HLA) mismatching contributes to liver allograft rejection, lower graft survival rates have been reported when HLA compatibility between donor and recipient is present[66-68]. To explain these apparently contrasting data, Mañez *et al*[69] evaluated 58 patients transplanted for HCV-related end stage liver disease and proposed a dualistic role of HLA in LT: HLA matching reduces ACR but increases the risk of RHC post LT, favoring a more efficient MHC-restricted antigen presentation, thereby increasing cell-mediated immune responses toward HCV-infected liver allografts. Langrehr *et al*[70] confirmed this observation in a larger retrospective analysis of 165 HCV-positive transplanted patients. The number of rejection episodes increased significantly in patients with more HLA mismatches ($P < 0.05$), whereas fibrosis progression (presumably related to RHC) was significantly faster in patients with 0-5 HLA mismatches compared with patients with a complete HLA mismatch. Globally, there is no correlation between number of HLA mismatches and graft survival. These data are in agreement with ours and other reports showing that MHC-I restricted T cells mighty be involved in the control of post-operative HCV spread[48,71]. In contrast, Belli *et al*[72], evaluated two separate cohorts of 120 and 190 patients with liver graft for HCV-related disease and found that HLA-DRB1 mismatch affected the risk of RHC and its severity, both in univariate analysis and, after correction for known clinical factors, in multivariate analysis. Similarly, Balan *et al*[73] demonstrated that HLA mismatching in the A locus significantly increases the rates of HCV recurrence.

More recently, in a retrospective study of 163 patients with documented post-LT RHC, Audet *et al*[74] could not find any relationship between the total score of HLA mismatches and HCV recurrence. On the contrary, a significant relationship between the individual scores of HLA mismatches and the recurrence of HCV were observed for some recipient HLA genotypes (HLA-A3, HLA-B35, HLA-DR3, HLA-DR7, HLA-DQ2 and HLA-DQ2-0)[74]. It should be noted that in the two studies above, different end points were used according to the length of follow-up, making them poorly comparable with each other. Furthermore, the differences regarding ethnic background, immunosuppressive protocols, HLA typing methods and the definition of hepatitis recurrence, particularly when protocol liver biopsies were not performed[75], make the comparison difficult.

Interleukin 28B

The association of allelic variation in the interleukin 28B (*IL-28B*) gene with HCV eradication after antiviral therapy provided new insight into the complex relationship between HCV and the human immune system. The initial report showed that patients homozygous for the C single-nucleotide polymorphism at position rs12979860 of chromosome 19q (corresponding to 3 kb upstream of the *IL-28B* gene) are twice as likely to achieve a sustained virological response to antiviral therapy than patients with either the CT or TT variant[76]. Subsequently, it was suggested that the CC variant is also associated with spontaneous viral clearance after acute HCV infection[77].

The evaluation of *IL-28B* gene in the setting of post-LT HCV infection is particularly complicated because recipients have two contributing sources of IL-28B genotypes: the recipient and the donor allograft. The impact of *IL-28B* on the antiviral treatment of HCV infection in the setting of LT goes beyond the purposes of this review, but has been fully analyzed[78]. Here, we focus on the impact of *IL-28B* in the spontaneous clearance of HCV and, if present, in the risk of ACR.

The spontaneous clearance of HCV infection is rare in the post-transplant setting, being limited to case reports. Only two studies of spontaneous clearance of HCV post-LT have included IL-28B genotyping, with three cases overall. Two out of the three cases involved recipients with the CT genotype, one had the CC genotype and all three patients received organs from CC donors[79,80]. Despite the limitations of the available data, it seems that the *IL-28B* gene of the donor has more influence than the recipient's in this setting. By contrast, Biggins *et al*[81] recently suggested favorable effects of the CC genotype in the non-transplant setting or when present in the recipient, but unfavorable when present in a donor liver graft.

Bitetto *et al*[82] assessed the risk of ACR in 251 consecutive patients undergoing LT (40% with HCV-related cirrhosis). They found a significantly lower risk of ACR in recipients with the CC genotype (20.6% with CC, 34.1% with CT and 47.8% with TT, $P = 0.003$), but the association was weaker in patients with HCV infection *vs* those with other etiologies of liver disease. Other authors have studied the association between ACR and IL-

28B SNPs in HCV-only cohorts, finding no significant variation in risk among the available genotypes for the *IL-28B* gene (donor and recipient)[83,84].

Overall, in this setting, the role of *IL-28B* gene remains uncertain.

Host immune response

There is evidence of a direct cytopathic effect of HCV in post-LT, which is supported by data demonstrating a higher viral load in patients with fibrosing cholestatic hepatitis with severe histology compared with patients with milder forms of HCV recurrence[14,85]. However, the evidence supporting a role for indirect immune-mediated mechanism in liver damage may even be more convincing.

In contrast to what happens in the vast majority of infections, specific antibodies to HCV, although diagnostic of infection, do not protect the host from subsequent damage from the same virus. Jain *et al*[86] quantified HCV antibody levels in 141 blood samples from 39 HCV-positive LT patients and confirmed that the antibody concentration did not correlate with viral load or hepatic injury in the post-LT setting.

In the non-transplant setting, immune responses appear to be crucial in the control of HCV infection. Patients with a self-limited course of acute HCV infection show activation of viral-specific CD4 and CD8 T-cells producing type 1 cytokines, such as interferon gamma (IFN-γ) and tumor necrosis factor-α (TNF-α)[87]. Presumably, the adaptive immune responses (CD4 helper T-cells and CD8 cytotoxic T-cells) and innate immune responses [natural killer (NK) and natural killer T (NKT)-cells] play a pivotal role in liver injury associated with RHC post-LT.

The majority of intrahepatic lymphocytes in patients with RHC after LT are represented by the CD8 T-cell subset: their presence is not proof of their role in liver injury, although it is unlikely that the predominant immune cells within the liver are simply bystanders. Asanza *et al*[88] demonstrated that patients with a more severe and progressive form of RHC after LT had higher numbers of activated lymphocytes, which implied that these activated CD8 T-cells play a critical role in injury and progression of liver disease. Corresponding to the main importance of CD8 T-cells in this setting, Rosen *et al*[71] described the presence of HLA-A2-restricted, HCV-specific CD8 T-cells in LT recipients, in whom the allograft was HLA-A2 positive, but the recipient was HLA-A2 negative. These cells are memory-effector recipient-derived T-cells that recognize HCV peptides uniquely in the context of HLA-A2. They are absent before the transplant, suggesting that the allograft is capable of selectively expanding naive CD8 T-cells that may function to control HCV spread in the allograft.

Evidence also suggest that not only CD8, but also CD4 T-cells play an important role in post-LT HCV recurrence.

Rosen *et al*[89] demonstrated that despite immunosuppression, HCV-specific, MHC class Ⅱ-restricted CD4 T-cell responses are detectable in patients with minimal histological recurrence after LT. By contrast, peripheral blood mononuclear cells from patients with severe HCV recurrence, despite being able to proliferate in response to non-HCV antigens, fail to respond to the HCV antigens. These findings suggest that the inability to generate virus-specific T-cell responses plays a contributory role in the pathogenesis of HCV-related graft injury after LT. Other authors reported that the HCV-specific CD4 T-cell response after LT occurs early, is multispecific, compartmentalizes to the liver and does not correlate with recurrent disease[90], while another study reported that robust CD4 T-cell immunity is associated with milder recurrence of HCV[91]. Mendler *et al*[92] evaluated peripheral blood CD4 T-cell ATP activity in an LT cohort and concluded that after LT, global cellular immune function appeared depressed at baseline in HCV-positive *vs* HCV-negative patients and remained significantly lower in case of RHC with respect to non-recurrence. This has been subsequently confirmed also by Te *et al*[93]. In addition, Alkhouri *et al*[94], using the same technique, revealed that a greater suppression CD4 T-cells was associated with more rapid progression of fibrosis in patients with RHC post-LT.

Other authors focused their attention on regulatory T-cells (Tregs) and their contribution to HCV disease. The Treg population, which accounts for 5%-10% of peripheral CD4 T-cells, constitutively expresses CD25[95] and can suppress host immune responses in the setting of autoimmune diseases, transplantation and antitumor immunity[96,97].

Carpentier *et al*[98] showed that CD4/CD25 Tregs are overexpressed, both peripherally and in the liver, in HCV-positive patients after LT, compared with HCV-negative patients. Moreover, Tregs were significantly overexpressed in patients with severe RHC compared with those with mild recurrence. These data agree with the findings of Perrella *et al*[99] who showed that transplanted patients with HCV recurrence show an increased frequency and function of CD4/CD25 Tregs, similar to patients with acute hepatitis C who develop persistent infection.

Although direct HCV infection of dendritic cells (DCs) is rare, HCV is associated with decreased numbers of peripheral DCs in patients with chronic HCV-related liver disease[100,101]; however, very limited data exist for the post-LT setting. Ocaña *et al*[102] studied two LT patients demonstrating an inadequate maturation of DCs with relapsing HCV infection. According to these preliminary data, Schvoever *et al*[103] studied a small series of 16 transplanted patients (eight of them HCV-positive) and showed a significant decrease in the relative and absolute values of blood DCs at day seven after LT compared with the values obtained before transplant. The number increased again one month later in both HCV-infected patients and controls. The authors suggested that this could partially explain the early and systematic

Table 1 Summary of the role of each immune cell line in recurrent hepatitis C post-liver transplant

HCV antibodies	No protective role against HCV reinfection	[86]
CD8 T-cells	Correlation with recurrent hepatitis C (RHC)	[71,88]
CD4 T-cells	Protective role against RHC	[89-94]
CD4/CD25 T-cells	Correlation with RHC	[98,99]
Dendritic cells	Defective in case of RHC	[102,103]
NK/NKT-cells	Controversial: defective in case of RHC; damaging role in RHC	[106-109]
Th1/Th2 paradigm	RHC is related to an imbalance towards Th2 prevalence	[98,102,110]

HCV: Hepatitis C virus; NK: Natural killer; NKT: Natural killer T.

recurrence of HCV infection in the liver graft.

More recently, because of their fundamental role in the spectrum of host immune responses in chronic HCV infection, greater attention has been given to NK and NKT-cells and the innate immune response. Studies suggest that NK and NKT-cells are involved in HCV clearance and in liver injury in the post-LT setting[104,105]. Rosen *et al*[106] demonstrated that patients who develop severe RHC after LT have a lower frequency of NK and NKT-cells in peripheral blood before LT, suggesting a protective role of these immune cells in the post-transplant period after exposure of the graft to HCV. Furthermore, they demonstrated that the presence of HCV infection is associated with impaired cytolytic activity of NK and NKT-cells, providing evidence for quantitative and qualitative defects in innate immunity associated with severe RHC after LT. Varchetta *et al*[107] analyzed the dynamics of NK-cells after LT and demonstrated a significant reduction of this subset of cells seven days post-LT, probably as a result of graft repopulation, returning to baseline values thereafter. Moreover, in contrast with Rosen, they revealed a significant correlation between expression of the natural cytotoxicity receptors on NK-cells and ALT ($P < 0.05$), supporting the hypothesis that NK-cells participate in the necro-inflammatory process. Recently, Howell *et al*[108] studied 70 patients with RHC post-LT and demonstrated an impaired function of NK-cells (comprising reduction of IFN-γ secretion) without impairment of NK-cell cytotoxicity in patients with rapid fibrosis.

Other authors evaluated KIRs (KIRs are a family of activatory and inhibitory receptors present on NK-cell surface interacting with self-MHC class I ligands) and demonstrated that the mismatching of KIR-HLA-C ligands between donor-recipient pairs is associated with recurrent hepatitis, and that the presence of KIR2DL3 in the recipient is correlated with fibrosis progression[109]. In fact, KIR-HLA disease association studies are intriguing, but complex and difficult to evaluate. The interpretation of these data is largely speculative and often based on simplified models of MHC-KIR functional interactions.

When considering the Th1/Th2 paradigm, RHC post-LT appears related to an imbalance towards Th2 prevalence and *vice versa*. Tambur *et al*[110] studied 68 LT recipients and found that among patients without RHC, the percentage of genetically low IL-10 (Th2-cytokine) producers was higher than among patients with RHC. Furthermore, a genetic tendency to produce higher levels of IFN-γ (Th1-cytokine) was noted among LT recipients with no RHC than among those with RHC. These findings have been confirmed by Ocaña *et al*[102], who described a loss of IFN-γ and TNF-α (Th1-cytokine) production in the LT recipient with relapsing HCV infection. In addition, Carpentier *et al*[98] suggested that high levels of IL-10 could be predictive of severe RHC post-LT.

Many data confirm the pivotal role of T-cells in the post-LT RHC setting, but they are essentially restricted to research field and are not usable in everyday clinical practice. A recent study by Nagai *et al*[111] appears particularly interesting because of its potential impact on daily clinical settings. They investigated the impact of peri-transplant absolute lymphocyte count (ALC) on HCV recurrence following LT in 289 patients and found that peri-transplant lymphopenia is significantly associated with higher rates of HCV recurrence. Furthermore, severe pre-LT lymphopenia appears to be an independent negative prognostic factor for overall survival. Therefore, the authors have proposed peri-transplant ALC as a novel and useful surrogate marker for prediction of HCV recurrence and patient survival, suitable for transplant physicians, surgeons and general practitioners.

A comprehensive summary of the role of each immune cell line is reported in Table 1.

IMMUNOSUPPRESSIVE TREATMENT AND IMMUNOTHERAPY

Corticosteroids

Corticosteroids are administered as an induction protocol during LT, and low doses combined with other immunosuppressants are used as maintenance immunosuppression after surgery. In cases of acute rejection, recipients receive pulse methylprednisolone to reverse the rejection.

In transplanted patients for HCV related liver disease, serum viral load increases very early post LT (typically by postoperative day two), during the induction steroid treatment[17,112], and methylprednisolone treatment for acute rejection leads to a 4-100-fold increase in serum HCV RNA[14,21]. Subsequently, the use of steroid boluses leads to an increased frequency of acute hepatitis, an earlier time to recurrence, a higher risk of progression to cirrhosis, and a higher risk of early post-transplant mortality[1,113,114]. Corticosteroids specifically increase HCV entry by upregulating factors like occludin and scavenger receptor class B type I ; therefore, the use of corticosteroids on HCV infection *in vivo* may cause increased HCV dissemination[115]. In addition, Boor *et al*[116] showed that

prednisolone suppresses the functions of plasmacytoid DCs (capable of producing IFN-α against HCV) by promoting their apoptosis.

However, despite the risks associated with steroid boluses, they remain the cornerstone of treatment for ACR, and corticosteroid maintenance therapy in association with newer immunosuppressive drugs has been evaluated significantly.

Klintmalm *et al*[117] considered 312 patients, randomized to one of three arms: tacrolimus (Tac) and corticosteroids *vs* Tac, corticosteroids and mycophenolate mofetil (MMF) *vs* Tac, daclizumab and MMF. They found no significant differences in graft or patient survival or HCV recurrence between the three groups; however, they found less risk of rejection in the corticosteroid-sparing arm. A subsequent study by the same group in 2011 showed there were still no differences in ACR, RHC, patient or graft survival at two years post-LT[118].

Kato *et al*[119] randomized 70 patients to Tac and; daclizumab *vs* Tac; and steroids *vs* Tac, MMF and daclizumab. They reported no significant difference in mean fibrosis stage between the three arms. Lladó *et al*[120] considered 198 patients randomized to basiliximab and cyclosporine with or without a 90-d prednisone taper, and reported similar fibrosis in the two groups. Both authors reported a reduction in bacterial infections and less post-transplant diabetes mellitus in the steroid-free groups.

Manousou *et al*[121] studied 103 patients and found that patients treated with Tac, azathioprine and maintenance steroids *vs* those not receiving maintenance steroids showed a lower incidence of severe fibrosis, suggesting a beneficial effect of maintenance steroids. Weiler *et al*[122] studied 30 HCV-positive patients who had received (after two weeks of Tac and corticosteroids) steroids *vs* placebo, in addition to Tac. They found that progression to cirrhosis was not influenced by continuing steroid therapy, but was more frequent in those receiving steroid boluses. Recently, Neumann *et al*[123] reported no significant differences in viral load, fibrosis score, or graft survival at 12 mo in 135 HCV-positive recipients randomized to Tac and daclizumab *vs* Tac and corticosteroids; however, these results appear inconclusive, mainly because of the higher dropout rates in the Tac and daclizumab group (55%) compared with the Tac and corticosteroids group (18%)[123].

Whether tapering-off of steroids might be more influential on outcomes than the avoidance or continued use of steroids is another matter of debate. Brillanti *et al*[124] studied 80 patients with RHC retrospectively and found that the slow tapering-off of steroids was the only factor associated with reduced recurrence and minor severity of post-transplant hepatitis C. Later Vivarelli *et al*[125] confirmed these data in a prospective randomized controlled trial, which showed that a rapid tapering (< 3 mo) is associated with more severe RHC. Finally, in 2008, Segev *et al*[126] performed a meta-analysis of 19 randomized trials that compared steroid-free with steroid-based immunosuppression. Although no individual trial reached statistical significance, the meta-analysis demonstrated that HCV recurrence is lower with steroid avoidance (RR 0.90, 95%CI: 0.82-0.99, $P = 0.03$). However, the authors themselves emphasized the heterogeneity of trials performed to date and, as such, did not recommend basing clinical guidelines on their conclusions.

Calcineurin inhibitors

Calcineurin inhibitors (CNIs) have been a cornerstone for immunosuppression since the National Institute of Health Consensus Conference approved LT for the treatment of end-stage liver disease in 1983[127]. Both cyclosporine A (CyA) and Tac bind with high affinity to a family of cytoplasmic proteins (called immunophilins), present in many immune cells. Immunophilin-dependent signal transduction *via* calcineurin leads to the activation of T-cell proliferation by regulating expression of the gene that encodes IL-2. The binding of CNIs blocks the activity of calcineurin and subsequently inhibits T-cell proliferation by the blockage of IL-2 production.

CyA has an antiviral effect against HCV: Watashi *et al*[128] showed an inhibitory effect of cyclosporin *in vitro* on HCV protein expression and replicon HCV ribonucleic acid levels, an effect that was not detected with Tac. Nakagawa *et al*[129] later confirmed these results; however, it remains unclear whether this finding reflects the *in vivo* situation.

Numerous retrospective studies have compared CyA with Tac in terms of the endpoints of patient/graft survival and HCV recurrence in HCV-positive recipients. Berenguer *et al*[130] reported a very comprehensive summary of 33 retrospective studies. In 28 studies, no consistent differences between CyA-based or Tac-based immunosuppressive regimens and recurrent disease were noted, while five studies suggested worse outcomes related with the use of Tac. In the same paper, the authors performed a meta-analysis on five prospective studies in the HCV-positive LT setting (366 patients), demonstrating that mortality, graft survival, acute rejection and fibrosing cholestatic hepatitis are comparable, independently of the CNI selected as the basic immunosuppressant. More recently, Irish *et al*[131] analyzed retrospectively data received from the United Network for Organ Sharing on 8809 HCV-positive LT recipients receiving either cyclosporine microemulsion (CSA ME) or Tac as maintenance immunosuppression. The results suggest that LT recipients receiving CSA-ME have an increased risk of death and graft loss because of HCV recurrent disease compared to those receiving Tac. These findings appear to contradict the above-mentioned previous results; indeed the explanation for the worse outcomes is not known. It may be related, however, to the higher rate of ACR and steroid-resistant ACR in the CSA-ME group: higher rejection rates could require multiple treatments of corticosteroid boluses, which are associated with more severe post-LT HCV recurrence.

MMF

MMF belongs to the class of anti-metabolite immunosuppressive agents. In addition to its potent immunosuppressive capacity, mycophenolic acid (MPA), the active metabolite of MMF, has an *in vitro* antiviral effect against HCV[132]. Moreover, in HCV cell culture models, MPA could induce the expression of important antiviral interferon-stimulated genes, probably involved in anti HCV activity[133]. Many studies have established that MMF monotherapy is ineffective because of unacceptably high incidences of ACR and chronic rejection[134,135]; therefore, in clinical practice, MMF is usually administered with lower doses of CyA or TAC, as a CNIs sparing agent, especially in cases of CNIs-related nephrotoxicity. In 2009, Germani *et al*[136] published a review based on 17 studies focusing the role of MMF in acute rejection and RHC. They showed that only two studies found a decreased severity of HCV recurrence with MMF, nine studies documented similar severities of HCV recurrence, and six studies showed increased severity of HCV recurrence. Subsequently, Manzia *et al*[137] showed, in a small retrospective study, a favorable effect of MMF monotherapy on the progression of liver fibrosis in HCV-positive LT patients.

Sirolimus

Sirolimus (otherwise named rapamycin, originally known as a macrolid antibiotic) inhibits the mammalian target of the rapamycin (mTOR) pathway by directly binding to the mTOR complex 1, resulting in blockage of cell cycle progression from the G1 to S phase, thereby causing inhibition of T-cell proliferation. It reduces transforming growth factor beta and procollagen, which are both important factors in the development of hepatic fibrosis; therefore, it has been proposed that immunosuppression with sirolimus could reduce fibrosis progression. In addition, sirolimus reduces the *in vivo* phosphorylation of NS5A phosphopeptides (which enhance HCV virus replication) and therefore might inhibit HCV replication[138]. Additionally, mTOR proteins were found to protect HCV against apoptosis; therefore, sirolimus might improve apoptosis of HCV infected hepatocytes[139]. There are few studies describing the role of mTOR inhibitors in HCV recipients and that confirm the data in the clinical setting. Wagner *et al*[140] studied 67 post-LT HCV-positive patients, 39 received a regimen including sirolimus and 28 patients received CNIs. The sirolimus patients showed a significant decrease in HCV RNA levels and a significantly higher survival rate than the CNIs cohort. Other studies demonstrated that sirolimus is associated with slower progression towards advanced fibrosis in transplanted patients with HCV recurrence, but did not find any effect on the timing or severity of post-transplant RHC[141,142]. Notably, the United States Food and Drug Administration has issued a black-box warning against the use of sirolimus in LT patients because of significantly higher rates of hepatic artery thrombosis, graft loss and death[143]. Moreover, recently, Watt *et al*[144] analyzed 26414 patients (12589 HCV-positive) in the American Scientific Registry of Transplant Recipients database, and found that the use of sirolimus is strongly associated with increased mortality in the HCV group, but not in patients without HCV. Thus, sirolimus should be used with great caution in HCV-positive LT recipients.

Other immunosuppressive agents

OKT3 is a monoclonal antibody targeted at the CD3 receptor, a membrane protein on the surface of T-cells. It is approved for the therapy of acute, glucocorticoid-resistant rejection of allogeneic LT but, unfortunately, the use of OKT3 is associated with early and severe RHC after LT[54].

Alemtuzumab (campath-1H) is a humanized, recombinant anti-CD52 monoclonal antibody that depletes circulating lymphocytes but spares stem cells. It has been used as an induction agent in LT; however, there is little data about its use in HCV-positive recipients. Many abstracts have suggested extreme caution when using alemtuzumab in HCV-positive liver recipients. This appears to be confirmed by Marcos *et al*[145], who studied a cohort of 38 HCV-positive recipients treated with alemtuzumab as an induction agent: they reported a low rate of patient and graft survival (71% and 70%, respectively) after a follow up of 14-22 mo.

Antithymocyte globulin (ATG) is a rabbit-derived polyclonal antibody directed against human thymocytes. It has been administered mainly as an immunosuppressive induction agent, with the intent of sparing steroids. Many studies have compared the impact of ATG *vs* steroids in post-LT HCV-positive patients, revealing no significant differences in terms of HCV recurrence and patient/graft survival[146-150]. De Ruvo *et al*[151] compared ATG and Tac *vs* Tac and steroids in HCV-positive liver recipients. They confirmed no difference in the rate of RHC; however, significantly lower HCV RNA levels were seen in the ATG arm. Finally, Uemura *et al*[152] evaluated the UNOS database, including 16898 adult primary LT patients who received ATG alone, ATG and steroids, daclizumab alone or steroids alone as induction immunosuppression. In the subgroup with HCV, the use of ATG with steroids was associated with significantly inferior graft survival compared with daclizumab alone or steroids alone.

Daclizumab and basiliximab are antibodies against the IL-2 receptor, originally developed in an attempt to reduce CNIs use in patients with renal dysfunction. To date, there have been few studies specifically in HCV-positive recipients. As above mentioned neither daclizumab[117-119] nor basiliximab[120] showed any effect on HCV recurrence post-LT. In a non-randomized study, Nelson *et al*[153] demonstrated that early RHC and more rapid histological progression was associated with the use of daclizumab.

Immunosuppression free state

In the long term, because of the important role played by immunosuppression in HCV recurrence patients, the goal is to utilize the least number of drugs at the lowest dose, while still providing effective immunosuppression. Yoshizawa *et al*[154] anecdotally reported two cases of living donor LT for patients with HCV-related cirrhosis who received right-lobe grafts from an identical twin, in which, thanks to genetic identity, no immunosuppressive drugs needed to be administered. HCV RNA kinetics showed a rapid increase following LT and liver biopsies performed one month after transplant showed acute lobular hepatitis in both cases. In the more common setting of LT without genetic identity, a permanent immunosuppression free state (IFS) can be achieved in almost 25% of cases[155]. Manzia *et al*[156] performed a meticulous review on this topic in 2012, evaluating globally 91 HCV-positive recipients included in immunosuppression withdrawal studies worldwide. Twenty-three HCV-positive patients (25%) achieved a sustainable IFS with more than one year of follow-up; and 19 of 23 (83%) did not show HCV recurrence/progression in the long term. The same authors recently reevaluated their own data on six HCV-positive recipients who completed 10 years of IFS follow up and demonstrated that maintaining IFS appears beneficial towards a reduction in fibrosis progression in the long term[157]. In conclusion, even though few studies have reported long-term outcomes of IFS in HCV-positive recipients, withdrawal of immunosuppression seems to have a favorable effect on HCV disease progression after LT, avoids side effects such as dyslipidemia and diabetes, and permits sparing of other drugs that might negatively impact the natural history of post-LT disease.

Immunotherapy

Adoptive immunotherapy has only been studied in a phase-1 trial. Lymphocytes extracted from liver allograft perfusate were able to generate an anti-HCV response, so that activated graft-derived NK-cells were isolated from the perfusate and injected intravenously into the transplanted recipients. Early data from the pilot study reported lower HCV RNA titers at one-month post-LT; however, the effect was not confirmed in the long term[158].

Prophylactic therapy with neutralizing antibodies is effective in patients transplanted for HBV-related liver disease; however, currently, there is no evidence that this strategy is effective in preventing HCV recurrence. HCV antibody therapy usually starts in the anhepatic phase and is then continued for 12 to 14 wk after LT. Gurusamy *et al*[159] performed a Cochrane meta-analysis on three trials comparing high dose HCV antibody *vs* low dose HCV antibody. No differences in patient and graft survival, virological response and fibrosis progression were observed. Discontinuation of therapy occurred in 35% of patients with the high dose antibody and in 17% of patients with the low dose antibody.

Recently, Chung *et al*[160] tested a human monoclonal antibody targeting the HCV E2 glycoprotein (MBL-HCV1) in a small pilot study (six patients). They demonstrated that this treatment delays median time to viral rebound compared with placebo treatment, even if it is not able to prevent it. Considering the lack of clinical benefit and occurrence of side effects, there is currently no evidence supporting the use of prophylactic HCV antibody treatment.

GRAFT-RELATED FACTORS

Living donor vs deceased donor

In Western countries, living donor LT (LDLT) is usually performed to decrease the mortality among patients awaiting transplant because of the shortage of donor organs. In Eastern regions, with low deceased donor organ availability, LDLT represents the standard of care for HCV end stage liver disease, with indications similar to those of deceased donor LT (DDLT) in Western world.

Early studies reported a worse graft outcome and earlier and more aggressive RHC after LDLT compared with DDLT[161-163]. To explain these findings it has been hypothesized that the more severe HCV recurrence in LDLT is related to the genetic similarity between donor and recipient[164] and that the intense hepatocyte proliferation that occurs in partial liver grafts may induce enhanced HCV replication[165,166].

Nevertheless, more recent studies did not confirm these findings, on the contrary, they often revealed improved results in LDLT recipients compared with DDLT, possibly because of the young age of the donor and shorter ischemic time of LDLT grafts[167-173]. Compared with LDLT, DDLT recipients usually also have a higher model for end stage liver disease score (MELD-score), which is considered an independent prognostic factor for severe RHC and worse patient/graft outcome; therefore, the above data should be evaluated with caution[174]. In agreement with these considerations, Jain *et al*[173], in a subanalysis of their study, adjusted for MELD score (< 25) and donor age (< 50 years), and revealed similar outcomes between LDLT and DDLT.

In light of that, LDLT appears to be recommended for HCV-positive patients, whenever it is available.

Donor age

The impact of donor age on outcome has become more and more important because of the increased use of liver grafts from older donors, reflecting the absolute shortage of available organs. Grafts from older donors are at greater risk of more severe HCV disease progression and impaired graft/patient survival compared with those from younger donors[175-179].

Lake *et al*[180] analyzed data from the American Scientific Registry of Transplant Recipients, looking at the effect of donor age on the outcome of 3463 HCV-positive transplanted patients. Donor age was the strongest predictor for graft loss in HCV-positive recipients, with

hazard ratios of 1.67 and 2.21 for donors > 40 years and > 60 years, respectively. In a multicenter study of more than 500 HCV-positive recipients, the risk of severe RHC following LT from a donor older than 60 years old was doubled in female compared with male recipients. This gender impact on HCV recurrence is not observed with younger donors and remains unexplained[181]. Recently, Avolio *et al*[182] analyzed 5946 liver transplants on a national Italian database and proposed that the MELD score adjusted by donor age (D-MELD: calculated as donor age × MELD) could accurately predict the outcome of HCV-infected recipients. In conclusion, it remains very difficult to define an age cut-off level beyond which older donors should not be used for HCV-positive recipients.

Grafts from HCV-positive donor

The increasing organ shortage prompted transplant centers to use grafts from HCV-positive donors. Several studies demonstrated that in HCV-positive recipients, grafts from HCV-positive donors are as safe as those from HCV-negative donors[177,183-186]. Wilson *et al*[187] evaluated data from the United Network for Organ Sharing (UNOS) and demonstrated that receiving a graft from an HCV-positive donor might be more favorable. They performed a case-control study (published only in abstract form and not in extenso) evaluating 38 HCV-infected recipients of HCV-infected grafts compared with 76 LT recipients of livers strictly meeting UNOS criteria. One-year patient survival rates of 97% favored recipients of HCV-infected grafts compared with rates of 87.5% for recipients of organs meeting the UNOS criteria. The same results have been noted for progression of fibrosis one-year post-LT: a 26% increase in fibrosis in HCV-infected organs compared with a 69% increase in the UNOS-approved group.

Nevertheless, considering the risk of super-infection and the impaired response of genotype 1 to antiviral treatment, it remains advisable that HCV-positive grafts should be used only in HCV genotype 1-positive recipients.

Graft steatosis

The impact of allograft steatosis on fibrosis progression and on the outcome of HCV-positive recipients remains controversial. Two studies indicated that moderate/severe donor graft steatosis (> 30%-35%) might induce more frequent, earlier and more severe HCV recurrence[188], and might contribute to fibrosis progression and poor outcome[189] post-LT. Nevertheless, Botha *et al*[190] found that recipients receiving grafts with mild steatosis (< 15% in their classification) had a good outcome, although only three out of 113 donors presented steatosis greater than 30%. Burra *et al*[191] reached the same conclusion, although they classified mild steatosis as < 30% and only five patients in their cohort presented steatosis > 30%. In light of that, the grade of steatosis seems to represent a crucial factor: grafts with mild steatosis are expected to be as safe as non-steatotic grafts.

Ischemia-reperfusion injury and ischemic preconditioning

Prolonged liver ischemia followed by reperfusion, which occurs during LT, results in severe injury that contributes to increased morbidity and mortality after LT. This phenomenon is defined as ischemia-reperfusion injury (IRI). IRI of the graft depends on many peri-operative factors: cold and warm ischemia time; preservation solution and technical factors during graft removal; donor status (cardiac or brain death); and type of reperfusion used. Its complexity depends on many variables; therefore, the majority of studies have found it difficult to focus on IRI. Within these limitations, ischemic injury to the graft seems to have a serious impact on patient/graft survival and disease progression in HCV recipients[192-194]. However, Killackey *et al*[195] reported a significant correlation between peak alanine transaminase and the severity of IRI on reperfusion biopsy among 477 HCV-positive recipients, but did not identify a correlation between the severity of IRI and the incidence or timing of HCV recurrence or incidence of ACR. When IRI is associated with moderate/severe steatosis (> 30%), the impact on graft survival becomes more and more important[188].

Liver ischemic preconditioning (IPC) is an endogenous mechanism consisting of brief and repetitive episodes of vascular occlusion, followed by reperfusion that makes the liver more tolerant to subsequent prolonged episodes of ischemia[196]. Several studies have demonstrated that IPC might have protective effects on IRI, but minimal or no clinical benefit[196,197] and a Cochrane systematic review confirmed this result[198]. No specific data exist for HCV-positive recipients.

CONCLUSION

In HCV-positive recipients, a balance between HCV, liver graft, recipient immune response and anti-rejection therapy is achieved in a few months after LT. During this period, almost all patients show an early graft reinfection, with HCV viremia reaching and exceeding pre-LT levels. Histological assessment for differentiating RHC from acute or chronic rejection is essential in this setting; however, differentiating the two pathological patterns remains difficult. The host immune response (mainly cellular mediated) appears to be crucial both in the control of HCV infection and in the genesis of ACR; however, it is also strongly influenced by anti-rejection immunosuppressive treatment. Currently, there is no clear immunosuppressive strategy to prevent HCV recurrence that could be strongly recommended for HCV-positive LT. Similarly, immunotherapy appears to be ineffective. It seems reasonable that ACR episodes and over-immunosuppression are more likely to enhance the risk of HCV recurrence through immunological mechanisms; therefore, both complete prevention of ACR and optimization of immunosuppression (possibly up to

IFS) should represent the main goals for reducing the rate of graft HCV reinfection. Other factors that might be modified by clinicians, include proper graft allocation and preservation injury to realize an ideal donor-to-recipient matching; however, many aspects related to these factors remain to be better determined in well-designed prospective studies. At present, post-LT HCV recurrence remains an unresolved thorny problem.

Evaluation of current treatment options for HCV in the transplant setting was not an aim of this review. Nevertheless, it should be stated that clinical concerns regarding HCV recurrence and needs of differentiation from rejection are strongly related to the available treatment options for the two conditions. Interferon-based treatments are unsatisfactory[199] and triple treatment with boceprevir and telaprevir is hampered by side effects and interaction with CyA and Tac[200]. In the near future, new drugs like sofosbuvir, that are better tolerated and with no interactions with CNIs, might represent the basis for reliable interferon-free treatment options for RHC[201]. Pre-emptive treatments to prevent HCV recurrence have been unsuccessful until now; however, newer drugs have the potential to change the natural history of HCV infection in transplanted patients.

ACKNOWLEDGMENTS

The authors wish to thank Ilaria Panzini, Francesca Cellauro and Andrew John Lawless for their valuable help in performing English proofreading.

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P- Reviewer: Boin IFSF, Sugawara Y **S- Editor**: Gou SX
L- Editor: Stewart G **E- Editor**: Zhang DN

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DOI: 10.3748/wjg.v20.i32.11116

World J Gastroenterol 2014 August 28; 20(32): 11116-11130
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (7): Liver transplant

Genetic variants of innate immune receptors and infections after liver transplantation

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Received: November 6, 2013 Revised: May 14, 2014
Accepted: June 12, 2014
Published online: August 28, 2014

Abstract

Infection is the leading cause of complication after liver transplantation, causing morbidity and mortality in the first months after surgery. Allograft rejection is mediated through adaptive immunological responses, and thus immunosuppressive therapy is necessary after transplantation. In this setting, the presence of genetic variants of innate immunity receptors may increase the risk of post-transplant infection, in comparison with patients carrying wild-type alleles. Numerous studies have investigated the role of genetic variants of innate immune receptors and the risk of complication after liver transplantation, but their results are discordant. Toll-like receptors and mannose-binding lectin are arguably the most important studied molecules; however, many other receptors could increase the risk of infection after transplantation. In this article, we review the published studies analyzing the impact of genetic variants in the innate immune system on the development of infectious complications after liver transplantation.

Key words: Innate immunity; Genetic variants; Single nucleotide polymorphisms; Liver transplantation; Post-transplant infections; Toll-like receptors; Mannose-binding lectin

Core tip: After liver transplantation, immunosuppressive therapy is needed to avoid allograft rejection that is mainly mediated through adaptive immunological responses. In the setting, the existence of genetic variants of innate immunity receptors may increase the risk of post-transplant infections in comparison with patients carrying wild-type alleles. This manuscript reviews the published studies analyzing the influence of innate immunity gene variants on the development of post-transplant infections and other complications.

Sanclemente G, Moreno A, Navasa M, Lozano F, Cervera C. Genetic variants of innate immune receptors and infections after liver transplantation. *World J Gastroenterol* 2014; 20(32): 11116-11130 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11116.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11116

INTRODUCTION

Liver transplantation is the treatment of choice for end-stage liver disease. New developments in surgical techniques, medical care, and immunosuppressant therapies have improved both graft and patient survival[1,2]. However, infections are still among the main complications after liver transplantation; it has been estimated that up to 80% of liver recipients will develop at least one episode of infection during the first year after transplantation[3,4]. Although several identifiable clinical risk factors are clearly associated with higher risk of post-transplant infection[5], variations in the receptors of the innate immune system could play an important role in its incidence and severity.

Bacteria are the leading cause of early infection after liver transplantation. Both the sources and etiology of infection change over time, according to the degree of immunosuppression and the presence of clinical risk factors. In the first month after transplantation, bacterial infections typically arise from the abdominal cavity, surgical wound, intravenous catheters, and the respiratory tract. Between the first and the sixth month after transplantation, the risk of opportunistic infections is increased because of the higher degree of immunosuppression. After the sixth month, infections are usually community acquired and predominantly respiratory and urinary, although cholangitis can occur if there are strictures in the biliary tree[3,6-8].

Viral infections after liver transplantation are frequent. Herpes simplex virus reactivation can occur early post-transplantation, typically with orolabial or genital ulcers appearing 2-3 wk after transplantation[9]. Herpes zoster virus reactivation occurs in around 10% of solid organ transplant recipients, but is mostly limited to dermal manifestations. The first year after transplantation poses the greatest risk of Epstein-Barr virus (EBV), which may be associated with lymphoproliferative disease[10]. Cytomegalovirus (CMV) can cause direct disease, manifested as fever, bone marrow suppression, and organ invasion. In addition, due to its ability to induce immunomodulation, CMV can cause indirect effects; these include favoring the development of opportunistic infections and hepatitis C virus (HCV) recurrence, EBV-associated lymphoproliferative disease, acute rejection, chronic allograft dysfunction, vascular and hepatic artery thrombosis, and ultimately, allograft failure and death[11-15]. Although several risk factors for CMV infection have been described, the most important is donor-recipient serology mismatch (donor positive/recipient negative) at the time of transplantation. Also, certain immunosuppressive therapies (lymphocyte-depleting drugs such as anti-thymocyte globulin) and acute allograft rejection are associated with a higher risk of CMV infection[11,16]. Other viral infections after transplantation, such as human herpesvirus 6 and 7, are less frequent and usually asymptomatic. However, they can also produce pneumonitis, encephalitis, hepatitis, and bone marrow suppression. Human herpesvirus 8 is also associated with Kaposi sarcoma[17].

Fungal infections represent one of the most life-threatening complications after liver transplantation. Although its incidence has declined (5%-30% depending on the series), they continue to be associated with high mortality. *Candida* species are the most frequent invasive fungi, followed by *Aspergillus* species[18]. Most invasive fungal infections (IFIs) occur early after transplantation, mainly during the first 3 mo. Multiple risk factors exist for IFI, such as pre-transplant comorbidity, surgical complications, and morbid post-transplant course[18-21]. Pre-transplant comorbidity includes a high Model for End-Stage Liver Disease score, acute hepatic insufficiency, pretransplant renal insufficiency, prolonged preoperative hospitalization, previous use of broad-spectrum antibiotics, fungal colonization, and re-transplantation[18-21]. Surgical complications include long surgical time, high intraoperative use of blood products, and choledochojejunostomy anastomosis, while a morbid post-transplant course involves dialysis requirement, acute rejection, CMV infection, early graft failure, and reoperation after transplantation[18-21].

The correct integrity and functionality of the host's immune system is a key pathogenic factor for the occurrence and severity of post-transplant infection. The innate immune system is the first line of defence against invasion by pathogens. It comprises cellular components [neutrophils, macrophages, dendritic cells, and natural killer (NK) cells] and molecular mediators (cell receptors, complement system, cytokines, and chemokines). Innate immune responses occur rapidly, with limited specificity and an inability to generate immunological memory. Innate immune receptors, also named pattern-recognition receptors (PRRs), are expressed by effector immune cells as either soluble or membrane-bound proteins. They recognize conserved structures, named pathogen-associated molecular patterns (PAMPs), which are broadly distributed among different types of microbes but absent from host cells, and are essential for microbial survival and pathogenicity. The binding of microorganisms by PRRs triggers intracellular signal pathways that culminate in the synthesis of cytokines and chemokines. This then causes inflammation, and induces the maturation and migration of antigen-presenting cells to secondary lymphoid tissues, where they activate adaptive responses. In contrast to the innate immune system, the adaptive immune system is slower to activate, but achieves highly specific immune responses based on immunological memory. Adaptive immunity is mainly mediated by T and B lymphocytes, which express antigen-specific receptors generated by genetic recombination during lymphocyte development. The repertoire of lymphocyte receptors is broad enough to recognize virtually any antigen. After the first exposure to an antigen, it takes up to 3-5 d to produce sufficient numbers of antigen-specific T and B cell clones, while the innate immune system generates a protective inflammatory response within minutes of pathogen exposure[22-26].

Acute cellular and humoral allograft rejection is mediated by T and B cells respectively[27-29]. Patients undergoing solid organ transplantation must receive immunosuppressive therapy, which predominantly alters the adaptive immune response by blocking lymphocyte activation signaling pathways, depleting lymphocytes, or diverting lymphocyte traffic[25,30,31]. In these circumstances, the innate immune response predominates in the defence against infection.

Gene polymorphisms, typically single nucleotide polymorphisms (SNPs), are common, occurring in > 1% of the general population. SNPs may alter the amino acid sequence, affect promoter characteristics, or may be completely "silent". Several SNPs have been described in relation to the genes encoding immune recognition[32]. Indeed, previous studies have found higher infection

rates in populations with SNPs in genes encoding innate immunity components[32-36]. Table 1 summarize the most relevant studies about innate immunity gene variants and risk of post-transplant liver infections.

INNATE IMMUNITY AND POST-TRANSPLANT LIVER INFECTION

Toll-like receptors

Biology: Toll-like receptors (TLRs) are a family of transmembrane proteins composed of a leucine-rich extracellular domain (the ligand binding site), a transmembrane domain, and a cytoplasmic domain [referred to as the TLR and interleukin (IL)-1 receptor (TIR) domain]. Binding of a PAMP to a TLR triggers a signaling cascade that ultimately induces the production of proinflammatory cytokines and type I interferons (IFNs). To date, 11 TLRs are described in mammals. Each TLR recognizes different pathogenic structures, and are expressed on the cell surface (*e.g.*, TLR1, TLR2, TLR4-6 and TLR10) or in endosomal compartments (*e.g.*, TLR3 and TLR7-9). When PAMPs are detected, TLR dimerization and recruitment of intracellular adaptor proteins and kinases occur. Most TLRs use myeloid differentiation primary response protein (MyD88) as the signal adapter, while TLR3 uses TIR-domain-containing adapter-inducing IFN-β (TRIF)[23,24,37].

TLR1 is associated with TLR2, and both recognize the microbial lipopeptides present in a wide variety of bacteria, fungi, parasites and viruses. To date, 17 polymorphisms have been described in the coding region, of which ten are non-synonymous, that is, they produce an amino acid change[38]. Some of these variants cause an inability of TLR1 to bind its agonist without diminishing its expression[39], while others result in reduced protein expression in the cell wall without reducing intracellular levels, suggesting an alteration of receptor trafficking[40]. In other cases, the polymorphism is associated with an excessive response that is partially mediated by increased cell surface expression of TLR1[41].

TLR2 recognizes microbial membrane constituents such as lipoteichoic acid, peptidoglycan, and lipoproteins of Gram-positive bacteria, lipoarabinomannan of *Mycobacteria,* and zymosan of *Candida*, among others. TLR2 needs to form heterodimers with TLR1 or TLR6 to be able to initiate cell activation. TLR2 sequencing has revealed multiple SNPs, although only a few are functionally relevant. The most frequently studied are Arg753Gln, Pro631His and Arg677Trp. The prevalence of these polymorphisms varies by ethnicity[42]. The Arg-753Gln polymorphism limits antigen recognition through deficient tyrosine phosphorylation rather than reducing protein expression, which impairs MyD88 recruitment, compromising TLR2-TLR6 assembly, and resulting in hyporesponsiveness to the antigen[43-48]. Defective membrane internalization and functional gain of the receptor has been observed with the Pro631His polymorphism, leading to increased immune activation[49].

TLR3 is an intracellular receptor located in the endoplasmic reticulum that typically recognizes double-stranded RNA of viral origin. After recognition of its ligand, TLR3 interacts with UNC-93B, a protein required for TLR3 trafficking from endoplasmic reticulum to the endosomal compartment[50]. At least 136 SNPs exist in the *TLR3*, of which only four exist in the protein-coding region and result in amino acid changes (N284I, Y307D, L412F and S737T). L412F is the most prevalent variant and reduces the receptor activity to near 30%, while Y307D and S737T have similar activity levels to the wild-type alleles, and N284I reduces the activity to background levels. These variants do not lead to a reduction in the intracellular protein expression or in vesicles, but do appear to alter the receptor trafficking to the cell surface[51,52].

TLR4 binds Gram-negative bacteria lipopolysaccharide (LPS), fungal mannans, and certain viral glycoproteins. First, LPS is bound by circulating LPS-binding protein (LBP), which functions as an opsonin for CD14, which in turn acts as a catalyst for the binding of LPS to MD-2, a co-receptor that is physically associated with TLR4. Finally, LPS binding to the TLR4/MD-2 complex activates intracellular signals that lead to the production of proinflammatory cytokines. Although various non-synonymous polymorphisms exist, only Asp299Gly and Thr399Ile are present at a frequency higher than 5%. They are located in the extracellular domain and, in Europe, frequently co-segregate[53]. Reduced responsiveness to LPS is higher in patients carrying the Asp299Gly polymorphism than in those with Thr399Ile. Some authors have demonstrated that hyporesponsiveness of *TLR4* variants is associated with a structural change in the ligand-binding receptor and a deficient recruitment of MyD88 and TRIF signalling adapters, but not with either decreased TLR4 expression or the interaction with MD-2 co-receptor[54,55].

TLR5 recognizes the flagellin of flagellated bacteria; of the 18 SNPs described, 13 are non-synonymous, and three reduce the functional response to bacterial flagellin. These variants are Asp694Gly, Leu822Phe and Arg392stop, but only the latter is present in > 10% of individuals. Arg392stop causes the loss of the transmembrane domain and the signaling of the entire cytoplasmic tail. TLR5 polymorphisms are associated with *Legionella pneumophila* infection and Crohn's disease[56,57].

TLR6 has a high sequence similarity to TLR1, and acts as a co-receptor with TLR2 that recognizes diacylated lipopeptides. However, information on *TLR6* polymorphisms is limited. Although 53 SNPs have been described, only 11 encode for changes in amino acid sequences, and only one has an allelic frequency > 5% (Ser-249Pro)[58]. The Ser249Pro polymorphism is associated with reduced IL-6 production in response to lipopeptide and mycobacterial stimulation. Although the mechanism by which this variant impairs IL-6 production is unknown, it seems that it is not associated with a reduction in protein expression levels[59].

TLR7 and TLR8, which share a high degree of struc-

tural similarity, are located in the endosomal compartment membranes, and recognize single-stranded RNA. TLR7 is mostly expressed in plasmocytoid dendritic cells, while TLR8 expresses predominantly in monocytes, macrophages, and myeloid dendritic cells. They facilitate the production of type I IFN and other cytokines. Little is known about *TLR7* and *TLR8* polymorphisms. The Leu11Gln variant of *TLR7* is the most prevalent, and impairs the signaling sequence. It has been associated with HIV, a higher susceptibility to HCV infection, and a lower response to IFN treatment[60-62]. The Met1Val polymorphism of *TLR8* leads to the formation of a truncated form of TLR8 that alters transcriptional activity. This variant has been associated with HIV and tuberculosis, and recent studies have shown an association of *TLR8* polymorphisms with HCV infection[63].

TLR9 is located in the endoplasmic reticulum where it detects bacterial and viral nucleic acids containing CpG motifs. At least 50 SNPs have been described, but most occur infrequently. Some of these variants are associated with noninfectious diseases such as lymphoma, asthma, and Crohn's disease[64,65], as well as infections such as HIV, malaria, bacterial meningitis, and tuberculosis[66-70].

TLR 10 and 11 have not been studied in depth. TLR10 is a member of the TLR1/2/6/10 cluster, and is hypothesized to have a similar function to TLR1 and TLR6, although the literature is scarce[49]. TLR11, which binds and recognizes uropathogenic bacteria, is probably nonfunctional in humans owing to a premature stop codon[53].

TLR polymorphisms and bacterial infection after liver transplantation: In a recent study that analyzed the genetic variants of a broad number of innate immune receptors in liver transplant recipients, including all TLR members, the authors found no association between genetic variants and clinically significant bacterial infections during the first 3 mo after transplantation[71].

The authors of a study of 706 liver recipients with *TLR4* polymorphisms failed to find an association between the Asp299Gly and Thr399Ile variants and either the incidence or outcome of Gram-negative infection; additionally, they noted that none of patients with *TLR4* variants developed septic shock[72]. Furthermore, *TLR4* variants were not associated with bacterial infections after either kidney or simultaneous kidney and pancreas transplantation[73]. These results contrast with previous published studies in immunosuppressed and immunocompetent patients. Lorenz *et al*[74] reported that patients admitted to intensive care units (ICUs) with septic shock, who carried the Asp299Gly *TLR4* polymorphism, were more likely to have Gram-negative infections and more severe disease. Agnese *et al*[75] also observed that, in patients admitted to a surgical ICU, those with *TLR4* polymorphisms had a higher incidence of Gram-negative infections. In the transplantation setting, Ducloux *et al*[76] reported a higher incidence of bacterial infection in kidney transplant recipients carrying the *TLR4* variant. Thus, there are discordant results on the influence of *TLR4* variants on Gram-negative bacterial infections. This is a research topic that warrants future investigation with larger cohort of patients.

Infections caused by Gram-positive bacteria are also important after liver transplantation[4]. Structural components of Gram-positive microorganisms are predominantly recognized by TLR2. Polymorphism of *TLR2* was first described following the observation of an increased risk of Gram-positive septic shock in patients admitted to the ICU with the genetic variant[77]. A study performed in 755 liver transplant recipients demonstrated that the Arg-753Gln *TLR2* polymorphism was not associated with an increased incidence of Gram-positive bacterial infections, although patients carrying the variant gene did present more frequently with septic shock and higher recurrence rates[78]. Despite this, the 90-d mortality was similar between patients carrying the variant and wild-type alleles.

Other studies have also reported that TLR polymorphisms are associated not with a higher incidence of infectious disease but with a more severe presentation. Specifically, individuals with sepsis and septic shock carrying *TLR1* variants have greater acute lung injury, organ dysfunction, and mortality, as well as a higher susceptibility to Gram-positive infection[41,79].

Solid organ transplant recipients are at higher risk of developing tuberculosis after transplantation, mostly by the reactivation of latent infection[80]. TLRs, specifically TLR2 (associated with TLR1 and TLR6), TLR4, and TLR9 play critical roles in recognizing mycobacteria[81]. Some studies have described an association between some of these polymorphisms and tuberculosis, although none are reported in liver transplant recipients. It is important to note that some TLR variants can be protective against mycobacterial infection[82].

TLR polymorphisms and viral infection after liver transplantation: The TLR2/TLR1 complex recognizes CMV envelope glycoproteins B and H, and associations between CMV infection and specific TLR SNPs have been described[46,83,84]. Kijpittayarit *et al*[85] studied the Arg-753Gln *TLR2* polymorphism in 92 HCV-infected liver transplant recipients, and observed that recipients carrying the variant allele had higher CMV DNA levels in their peripheral blood when compared with recipients carrying the wild-type allele. Regardless of the higher CMV replication in patients carrying the *TLR2* variant allele, only homozygous patients presented CMV disease more frequently. In a later study of 737 liver recipients published by the same group, an analysis of the association between *TLR2* polymorphisms and CMV infection revealed that homozygous Arg753Gln was significantly associated with an increased risk of CMV disease, particularly tissue-invasive forms[86].

Other viral infections have also been related to deficiencies of innate immunity, but no studies were performed in liver transplant recipients. The herpes viruses are known to be recognized by TLR2, TLR9 and TLR3.

Table 1 Main published findings in the association of innate immune gene variants with the development of infections after liver transplantation

	Innate immune receptor polymorphism	Results	Ref.
Bacterial infections	Donor MBL	Incidence of CSI was 3.8-fold higher in the recipients of MBL variant livers	Bowman *et al*[123]
		Mutation in the donor MBL2 was associated with CSI (HR = 2.8, P = 0.02)	Worthley *et al*[124]
		Mutation in donor MBL2 was associated to CSI (HR = 2.58, 95%CI: 1.62-4.10)	de Rooij *et al*[126]
		Higher incidence of septic shock in recipients of a MBL2 variant liver (HR = 9.64, 95%CI: 2.59-36)	Cervera *et al*[127]
	Donor ficolin	Mutation of donor ficolin was associated to CSI (HR = 2.33, 95%CI: 1.36-4)	de Rooij *et al*[126]
	NOD2	NOD2 polymorphism was associated to CSI (HR = 2.0, P = 0.04)	Janse *et al*[145]
	Donor MASP	Wild-type allele of MASP2 in the donor was associated to CSI (HR = 2.65, 95%CI: 1.22-5.73)	de Rooij *et al*[126]
	TLR2	Patients with TLR2 polymorphism presented higher rates of Gram positive infection recurrence (27.8% *vs* 11.8%, P = 0.07) and gram positive septic shock (11.1% *vs* 1.2%, P = 0.047)	Lee *et al*[78]
Viral infections	TLR2	CMV load was higher in patients with TLR2 polymorphism (P = 0.03)	Kijpittayarit *et al*[85]
		CMV disease was higher in patients homozygous for the TLR2 polymorphism (HR = 1.91, 95%CI: 0.91-3.4)	
		TLR2 polymorphism homozygosity was associated to tissue-invasive CMV disease (HR = 3.40, 95%CI: 1.51-7.64)	Kang *et al*[86]
	MBL	MBL wild-type genotype was associated to a higher incidence of CMV invasive disease in SOT (OR = 6.0, 95%CI: 1.1-32.5)	Cervera *et al*[129]
		MBL deficient donor is associated to CMV infection (54% *vs* 32%, P = 0.02)	de Rooij *et al*[130]
	Ficolin	44% CMV infection in patients receiving a FNC2 wild-type liver *vs* 27% in patients receiving a variant FCN2 liver (P < 0.02)	de Rooij *et al*[130]
Fungal infections			No studies in liver transplantation
HCV recurrence	TLR2	Homozygous TLR2 mutation is associated with allograft failure and mortality in HCV-infected recipients (RR = 5.2, 95%CI: 1.65-13.9)	Eid *et al*[155]
	TLR3	Higher rate of allograft failure and mortality in patients with TLR3 polymorphism (44.3% *vs* 30.8%, P = 0.09)	Lee *et al*[156]
		HCV patients with rapid fibrosis progression had impaired TLR7/8-induced interferon response compared with patients with slow fibrosis progression (P = 0.039) and impaired TLR3 and TLR9 cytokine production (P = 0.008)	Howell *et al*[157]
	NK cells	Lack to antiviral response to HCV therapy associated to the absence of the activating NK receptor haplotype KIR2DS2 (P = 0.008). KIR2L3 haplotype has been correlated to recurrent allograft hepatitis (P = 0.04)	Nellore *et al*[151]
	IL28B	No difference in the frequencies of IL28B polymorphisms in patients with and without fibrosing cholestatic hepatitis	Duarte-Rojo *et al*[168]
		Recipients with CC genotype or CT genotype had delayed time to HCV recurrence compared to TT (10.4 *vs* 6.7 mo, P = 0.002). Recipients with TT genotype had worse graft survival (42% *vs* 62%, P = 0.02)	Allam *et al*[162]
		Higher response to antiviral therapy for CC genotype compared to CT or TT (59% *vs* 25%, P = 0.002). Higher sustained virological response in patients with favorable donor and recipient genotypes (P < 0.01)	Coto-Llerena *et al*[161]
		Higher progression to cirrhosis (HR = 5.96, 95%CI: 1.29-27.6), liver-related death or re-transplantation among recipients with a CC genotype donor.	Duarte-Rojo *et al*[167]
		IL28B genotype in the recipient is associated to severe HCV recurrence (OR = 4.27, P = 0.014). Allele IL28B T in the donor tend to have lower incidence of severe HCV recurrence (OR = 0.46, P = 0.19)	Cisneros *et al*[166]
		Sustained viral response to HCV therapy was 100% if both donor and recipient were CC genotype, while it was only 25% if neither donor nor recipient had CC genotype (P = 0.025)	Firpi *et al*[163]
		IL28B non-CC in the recipient had a higher risk of severe recurrent HCV (OR = 1.57, P < 0.05). IL28B CC in the donor was associated to higher risk of severe recurrent HCV (OR = 7.02, P < 0.001)	Biggins *et al*[164]
No association	TLR	None of a broad range of genetic variants in recipient and donor innate immunity receptors was associated to bacterial or fungal infections after liver transplantation.	de Mare-Bredemeijer *et al*[71]
	MBL	The presence of donor MBL2 variant is not associated to a higher incidence of CSI (47% *vs* 36%, P = 0.19)	Curvelo *et al*[125]
	TLR4	Incidence of Gram-negative infection was not higher in patients with TLR4 mutations (13.5% *vs* 19.3% in patients with wild-type allele, P = 0.39)	Lee *et al*[72]
	TLR2	Incidence of Gram-positive bacterial infection was not different related to TLR2 polymorphism (31.6% *vs* 31.6%)	Lee *et al*[78]

NOD: Nucleotide-binding and oligomerization domain; TLR: Toll-like receptor; MBL: Mannose-binding lectin; CSI: Clinically significant infections; SOT: Solid organ transplant; MASP: MBL-associated serine proteases; NK: Natural killer; IL: Interleukin.

TLR2 polymorphisms have been associated with a higher recurrence rate of herpes simplex virus (HSV) type 2 genital ulcers, and greater viral shedding in healthy individuals[87]. Recurrent herpes labialis also appears more frequent in individuals with a deficient TLR3 response, which is probably related to the *L412F* polymorphism[88]. In contrast, Svensson *et al*[89] observed that individuals with the same SNP had lower HSV2 infection rates. Varicella-zoster virus is also recognized by TLR2[90].

TLR polymorphisms and fungal infection after liver transplantation: TLR2, TLR4 and TLR9 reportedly mediate some aspects of fungal recognition[91].

Invasive candidiasis: A study in mice observed that cytokine production in response to candidal infection was determined by TLR2, but that TLR4 also participated in the host defense by modulating chemokine synthesis and neutrophil recruitment[92]. Specifically, TLR2 has been observed to recognize phospholipomannan, while TLR4 recognizes O-linked mannan. TLR9 recognizes *Candida albicans* DNA and induces cytokine production, but the role of TLR9 in invasive candidiasis might only be secondary[93]. In a study performed in non-neutropenic patients, Van der Graaf *et al*[94] described that the presence of the Asp299Gly and Thr399Ile *TLR4* polymorphisms was associated with increased risk for candidal septicemia. Woehrle *et al*[95] studied the cytokine response in critically ill patients with septic shock and its relationship with *TLR2* polymorphisms. The authors found that patients with candidal septicemia in the presence of the Arg753Gln *TLR2* SNP had an attenuated cytokine production when compared with patients with the wild-type allele. More recently, Plantinga *et al*[96] analyzed the SNPs related to TLR1, TLR2, TLR4, TLR6, TLR9, MyD88 and and another adaptor protein named TIRAP (Toll-interleukin 1 receptor domain containing adaptor protein) in patients with candidal septicemia, and they only observed an increased susceptibility to candidemia in patients with *TLR1* polymorphisms. No information exists about the risk of candida infection and TLR polymorphisms following liver transplantation.

Invasive aspergillosis: Initial *in vitro* studies observed TLR2 to be the critical receptor for *Aspergillus* spp. recognition by the innate immune system[97], and that this was mediated by CD14. Subsequent studies have determined that TLR4 can also detect *Aspergillus*, but that this only induces cytokine production in response to *Aspergillus* conidia, and not to the hyphae that are responsible for tissue invasion[98-100]. More recently, TLR9 has been observed to recognize *Aspergillus* DNA[101]. Studies in stem cell recipients have described an association between TLR polymorphisms and invasive aspergillosis. For example, Bochud *et al*[102] analyzed TLR2, TLR3, TLR4 and TLR9 polymorphisms in 336 patients undergoing allogeneic hematopoietic stem-cell transplantation; of whom, 33 developed invasive aspergillosis. The authors found an association between donor *TLR4* polymorphisms and a higher risk of invasive aspergillosis. Recently, de Boer *et al*[103] described similar results in patients receiving allogeneic stem cell transplantation from donors with TLR4 polymorphisms. TLR2 can recognize *Aspergillus,* and TLR2 ligand recognition usually occurs through heterodimeric association with TLR1 or TLR6. Therefore, Kesh *et al*[104] analyzed the association between *TLR1* or *TLR6* polymorphisms and the incidence of invasive aspergillosis in stem cell transplantation recipients, and identified that either the Arg80Thr *TLR1* polymorphism or the combination of *TLR1* Asn248Ser and *TLR6* Ser249Pro polymorphisms in the recipients were associated with invasive aspergillosis. In the setting of liver transplantation, no studies have analyzed the association of TLR polymorphisms with the incidence of invasive aspergillosis.

Other invasive fungal infections: *Pneumocystis jirovecii* pneumonia is a potentially life-threatening pulmonary infection in immunocompromised patients. Its incidence has declined substantially with the use of universal prophylaxis[105]. The major host defense system against *Pneumocystis* infection is adaptive immunity, in which $CD4^+$ T cells are the most important. In *TLR4*-deficient mice infected with *Pneumocystis*, the authors observed that the number of lung cysts did not differ between *TLR4*-deficient and wild-type mice, but they did observe that the former had more lung destruction[106]. The authors concluded that TLR4 signaling was not protective against *Pneumocystis* infection, but was responsible for regulating inflammation after infection. Zhang *et al*[107] analyzed the role of TLRs in the recognition of *Pneumocystis* in a mouse model, revealing that cytokine production in alveolar macrophages was activated through recognition by TLR2, but not TLR4. In a subsequent study, they also reported that TLR2 was not involved in the phagocytosis of *Pneumocystis*, but that *TLR2* deficient mice had increased microbial burden when compared with wild-type mice[108]. A recent study in mice found that cytokine production in response to *Pneumocystis* infection was dependent on MyD88, but that it was independent of both TLR2 and TLR4[109]. In conclusion, it is not clear which receptors are involved in the recognition of *Pneumocystis*.

The incidence of cryptococcal infection after liver transplantation is low, and usually occurs in the late post-transplant period because of reactivation of latent infection. Mortality increases when the central nervous system is involved. Additionally, liver transplantation is associated with a more severe presentation, higher risk of dissemination, and a poorer outcome than other transplant types[110]. Host defence against *Cryptococcus neoformans* is mainly mediated by $CD4^+$ T lymphocytes, while the MyD88 adaptor plays a critical role in the innate immune response against *Cryptococcus*. Recent studies demonstrate that TLR9 recognizes the DNA of this fungal pathogen[111,112]. Previous studies have reported that, although glucuronoxylomannan is a ligand for TLR2 and TLR4, it seems that these receptors are dispensable for the defence against *Cryptococcus*. van der Graaf *et al*[113] and Yauch *et al*[114] analyzed mononuclear cells from volunteers, and observed that individuals carrying the Asp299Gly *TLR4*

polymorphism did not develop increased tumor necrosis factor-α or IL-10 levels when their mononuclear cells were stimulated by *Cr. neoformans*.

Mannose-binding lectin

Mannose-binding lectin (MBL) is a soluble C-type lectin that recognizes carbohydrates on the surface of numerous microorganisms, including *N*-acetylglucosamine *D*-mannose, *N*-acetyl mannosamine, and *L*-fucose. Although it is primarily synthesized by the liver, small levels of extrahepatic production have been described in the small intestine and testes, which represent about 1% of the total produced. MBL circulates as a serum protein, although an intracellular pool of MBL exists. It consists of a structural subunit composed of three identical polypeptide chains forming a triple helix. Circulating MBL consists of oligomers of this subunit, with higher order oligomers (tetramers to hexamers) being the effective forms. Additionally, MBL-associated serine proteases (MASPs) are present in the serum, of which only MASP-2 effectively activates the complement cascade. MBL can facilitate microorganism phagocytosis through direct opsonization or triggering complement activation, but also cooperates with other PRRs[115,116]. The MBL gene (*MBL2*) is located at chromosome 10q11.2-21. There are five known polymorphic sites within the *MBL2* gene, all of which decrease the amount of circulating MBL. Two SNPs are situated in the *MBL2* promoter region (H or L at -550 and Y or X at -221), and three are in exon 1, at codons 52 (allele D), 54 (allele B) and 57 (allele C). These SNPs interfere with the formation of higher-order oligomers and cause decreased serum levels of MBL. Variant alleles of both codon 54 and 57 reduce the levels of functionally viable MBL in the serum to approximately one-eighth that of the wild-type phenotype. The variant allele at codon 52 encodes intermediate levels of MBL, and the polymorphism in the promoter region also reduces the MBL levels[34,117].

The presence of MBL variant alleles is associated with bacterial and viral infection in both immunosuppressed and immunocompetent patients. In some studies, variant alleles have resulted in more severe clinical infections. In contrast, intracellular infection appears to be more frequent in individuals with high MBL levels, because of the increased opsonization and phagocytosis. Low levels of MBL might mitigate the excessive complement-mediated damage found in inflammatory conditions that cause tissue destruction[33,118-122].

In the liver transplantation setting, because MBL is mainly produced by the liver, serum MBL levels depend on the donor genotype after transplantation. Thus, reduced serum MBL levels are seen in recipients with the wild-type *MBL2* genotype who receive a liver with an *MBL2* variant genotype. Conversely, patients with an *MBL2* variant genotype receiving a liver from a wild-type donor, experience increased serum MBL levels after transplantation. These changes occur during the first 2 d after transplantation[123,124].

***MBL2* polymorphisms and bacterial infection after liver transplantation:** Although a recent study did not observe a higher incidence of bacterial infection in recipients of *MBL2* variant livers[125], several studies have described a higher frequency of clinically significant bacterial infections in patients receiving livers from *MBL2* variant donors compared to patients receiving wild-type livers[123,124,126]. In addition, these studies observed that *MBL2* polymorphisms in the recipient were not associated with increase rates of infection. A study by our group found no association between *MBL2*-deficient livers and the incidence of bacterial infection, but we did observe that recipients of an *MBL2* variant allograft had more severe infection (more frequent septic shock, higher levels of C-reactive protein, and higher creatinine levels)[127].

***MBL2* polymorphisms and viral infection after liver transplantation:** The first study relating CMV infection to MBL deficiency in transplantation involved 16 kidney recipients at high risk of developing CMV infection (donor positive/recipient negative). The authors observed that patients with low serum MBL levels had a higher incidence of CMV infection[128]. The same results were obtained in a study of kidney and pancreas recipients[86]. More recently, we determined that the presence of a wild-type genotype was associated with a higher incidence of invasive CMV disease in recipients with positive CMV serology pre-transplantation. These results could be explained by the facilitation of CMV phagocytosis in patients with higher serum MBL levels[129]. In liver transplantation, de Rooij *et al*[130] described that patients receiving livers from *MBL2* deficient donors had an increased risk of CMV infection compared with those receiving a wild-type liver.

MBL does not neutralize HSV1 and HSV2, which block the activation of both the classical and alternative complement pathways through glycoprotein C[131]. Despite this, Seppänen *et al*[132] described that individuals with recurrent HSV2 infection presented the *MBL2* variant genotype more frequently.

***MBL2* polymorphisms and fungal infection after liver transplantation:** MBL binds to *Aspergillus fumigatus*, *C. albicans,* and *Cr. neoformans*[133]. There are no studies relating *MBL2* polymorphisms with the incidence of fungal infection in liver transplant recipients, but some studies have described associations in other immunosuppressed patients. For example, Granell *et al*[134] observed that donor with an MBL-low genotype resulted in more invasive fungal infections in HLA-identical allogeneic stem cell transplantation recipients. Lambourne *et al*[135] reported that immunocompromised patients with lower MBL levels had a higher incidence of invasive aspergillosis. Ou *et al*[136] reported a higher incidence of cryptococcal meningitis in non-HIV patients with *MBL2* polymorphisms. Additionally, in nonimmunosuppressed patients with secondary peritonitis, candidal infection was more frequent in those with low MBL levels[137].

As stated, MBL is associated with MASPs. When MBL recognizes a ligand, one of three MASPs is activated. Of these, MASP-2 plays a predominant role in complement activation. The *MASP2* gene is located on chromosome 1p36.23-31, where nine polymorphisms have been identified[138-140]. de Rooij *et al*[126] observed that *MASP2* polymorphism homozygosity in the donor was associated with an increased incidence of clinically significant bacterial infections in liver transplant recipients. In stem cell transplantation, Granell *et al*[134] described that *MASP2* mutations in the recipient were associated with higher incidences of invasive fungal infection.

Other PRRs

L-ficolin binds to carbohydrate present in lipoteichoic acid, a constituent of Gram-positive bacteria[141]. Similar to MBL, the liver synthesizes ficolin, and polymorphisms in the promoter region of the *FCN2* gene are associated with differences in ficolin-2 serum levels. Patients receiving a donor liver with *FCN2* polymorphisms had demonstrated an increased incidence of clinically significant bacterial infection[126]. Liver recipients of donors without the minor T-allele of the *FCN2* gene present a higher incidence of CMV infection compared with patients receiving a liver with at least one copy of the minor T allele. The presence of an *FCN2* variant in the recipient does not increase the incidence of CMV infection[130]. Recently ficolin-A has been demonstrated to bind *Aspergillus* conidia, but there are no studies relating ficolin deficiency with invasive fungal infections in liver transplant recipients[142].

Nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) are a family of intracellular receptors that recognize bacterial peptidoglycans. They are expressed by macrophages, dendritic cells and certain intestinal epithelial cells. NLR subfamilies include the NOD receptors (NOD1 and NOD2). Three *NOD2* SNPs have been described, and the variants have been associated with inflammatory disease such as arthritis, asthma, and Crohn's disease[143,144]. Furthermore, a recent study has described a higher incidence of bacterial infection in liver transplant recipients carrying the R720W polymorphism[145]. The authors also observed that homozygous recipients for this polymorphism developed earlier infection than heterozygous or wild type. However, this result has to be interpreted with caution given the small number of patients.

Dectin-1 is a C-type lectin receptor expressed predominantly in the lungs and intestine by dendritic cells, monocytes, macrophages, neutrophils, a subset of T cells, B cells, eosinophils, and mast cells. It is the main receptor involved in the recognition of β-glucans, major structural components of the fungal cell wall, and interacts with *Candida*, *Aspergillus*, *Pneumocystis*, *Coccidioides*, *Penicillium,* and *Saccharomyces*[98,146,147]. Ferwerda *et al*[148] first described an association between the Dectin-1 polymorphism and a higher incidence of mucocutaneous fungal infection. In a recent study with hematology patients, a Dectin-1 polymorphism was associated with increased risk of invasive aspergillosis and higher levels of galactomannan[149]. In contrast, Rosentul *et al*[150] found no association between Dectin-1 polymorphisms and higher incidences of candidemia or worse clinical outcomes.

INNATE IMMUNITY AND HCV RECURRENCE

HCV infection is the most frequent cause of end-stage liver failure requiring transplantation. After transplantation, HCV recurrence occurs in nearly all patients and the progression to cirrhosis and allograft failure is often accelerated. Between 10% and 30% of liver recipients develop allograft cirrhosis within 5 years of transplantation[151,152]. Risk factors associated with an accelerated progression to cirrhosis include: high HCV RNA load, genotypes 1b and 4 (probably related to the lower response to antiviral treatment), female gender, older donor age, steatosis of the graft, the degree of HLA matching, the immunosuppressive drugs used, and CMV and human herpesvirus 6 infection after transplantation[153,154]. However, genetic factors may also play an important role in HCV recurrence and subsequent graft loss.

In a study performed in 92 HCV-infected, liver transplant recipients, the authors analyzed the relationship of *TLR2* Arg753Gln, *TLR4* Asp299Gly and Thr399Ile polymorphisms with HCV recurrence, liver fibrosis, and mortality. They described a higher incidence of allograft failure and mortality due to recurrence of HCV infection in individuals homozygous for the *TLR2* polymorphism, but not in either the *TLR2* heterozygous patients or those with *TLR4* variants[155]. The same team recently described that TLR2-deficient cells were unable to respond to HCV core and NS3 proteins *in vitro*, because the interaction between TLR2 and the intracellular MyD88 adapter was defective[47].

In a study analyzing the relationship between the Phe412Leu *TLR3* polymorphism and HCV infection in liver transplant recipients, the *TLR3* polymorphism occurred more frequently in HCV-infected liver transplant recipients than in recipients for other indications. Univariate analysis uncovered a higher incidence of allograft loss and mortality in HCV-infected patients with the *TLR3* polymorphism when compared with the wild-type genotype, although this association was lost following multivariate analysis[156]. Howell *et al*[157] recently analyzed several TLR polymorphisms and their relation with rapid fibrosis after liver transplantation in HCV infected patients. They concluded that patients who developed fibrosis earlier after transplantation were more likely to have deficient TLR7/8 and TLR3 responses.

Variants in NK cell receptors are also associated with the risk of HCV recurrence after liver transplantation. Serum NK cell levels prior to transplantation may predict the severity of HCV recurrence[151].

Although previous studies have described associations between HCV recurrence and distinct innate recep-

tors, most have found a clear relation with the IL-28B polymorphism. IL-28 comprises a family of cytokines (type Ⅲ IFN) including IL-28A, IL-28B and IL-29. The IL-28B gene (*IL28B*) is located on chromosome 19, is composed of six exons, and produces IFN-λ3, which regulates T regulatory cells and enhances cellular adaptive immunity[158,159]. Polymorphisms in this gene do not affect serum IL-28B transcript levels, and their impact on IL-28B function remains unknown. IL-28B is produced by both bone-marrow-derived cells and hepatocytes. Thus, the interplay between donor and recipient genotypes is complex after liver transplantation. In nontransplanted patients infected with HCV, *IL28B* polymorphisms have been associated with a sustained virological response after antiviral treatment; predominantly in those patients infected with viral genotype 1. In addition, the existence of CC genotype in the rs12979860 locus has been associated to spontaneous HCV clearance[160].

In the liver transplantation setting, various studies have analyzed *IL28B* polymorphisms, mainly rs12979860 (alleles T and C) and rs8099917 (alleles T and G). Recipients with the *IL28B* rs12979860 TT genotype typically have early and more severe HCV recurrence, and a higher incidence of graft loss. Non-CC recipients who received a liver from a CC donor had the highest risk of developing severe HCV recurrence. Patients with CC genotype not only had less severe HCV recurrence, but also presented higher rates of sustained viral response after antiviral treatment when compared with recipients with a different genotype. Although patients who received a liver from a CC donor have more risk of severe HCV recurrence, they also have higher virological response rates than patients receiving a liver from a non-CC donor[8,161-167]. The rs8099917 IL28B polymorphism has also been studied in patients with liver transplantation, revealing that non-TT recipients receiving a liver from TT donors had the highest risk for severe recurrence. The *IL28B* genotype had no effect on graft survival in liver recipients without HCV infection[164].

Some authors have tried to analyze the relation between *IL28B* polymorphisms and the development of fibrosing cholestatic hepatitis (a severe manifestation of recurrent HCV infection after liver transplantation). As this is an infrequent complication, the results must be interpreted with caution. However, it seems that recipients with an unfavorable *IL28B* genotype tend to have more fibrosing cholestatic hepatitis, and that this complication is more frequent when the donor has a favourable *IL28B* genotype[168].

CONCLUSION

The innate immune system could play an important role in liver transplantation. The majority of studies have demonstrated that SNPs of the innate receptors are associated with higher infection rates after transplantation. The importance of these results is in the possibility of establishing individualized risk profiles for each patient prior to transplantation and the development of prophylactic strategies after transplantation. Furthermore, if specific deficiencies can be proven to be associated with higher infection rates, it may be possible to use recombinant molecules (*i.e.*, recombinant MBL) as therapeutic agents. Future studies on the association between innate immunity variations and the risk of infection after liver transplantation are warranted.

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P- Reviewer: Biswas T, Ikemoto T **S- Editor**: Qi Y
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World Journal of Gastroenterology

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DOI: 10.3748/wjg.v20.i32.11131

World J Gastroenterol 2014 August 28; 20(32): 11131-11141
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (7): Liver transplant

Hepatic hemodynamic changes during liver transplantation: A review

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Received: January 3, 2014 Revised: March 21, 2014
Accepted: April 30, 2014
Published online: August 28, 2014

Abstract

Liver transplantation is performed in the recent decades with great improvements not only technically but also conceptually. However, there is still lack of consensus about the optimal hemodynamic characteristics during liver transplantation. The representative hemodynamic parameters include portal vein pressure, portal vein flow, and hepatic venous pressure gradient; however, there are still others potential valuable parameters, such as total liver inflow and hepatic artery flow. All the parameters are correlated closely and some internal modulating mechanisms, like hepatic arterial buffer response, occur to maintain stable hepatic inflow. To distinguish the unique importance of each hepatic and systemic parameter in different states during liver transplantation, we reviewed the published data and also conducted two transplant cases with different surgical strategies applied to achieve ideal portal inflow and pressure.

Key words: Liver transplantation; Hemodynamics; Graft inflow modulation; Liver circulation; Small-for-size syndrome

Core tip: Even with the technical advancement of liver transplantation, there is still lack of consensus about the optimal range of the hepatic hemodynamic parameters intra-operatively. In this article, we review the physiology of liver hemodynamics in the normal population and also in the cirrhosis-related portal hypertension. The hemodynamic changes during liver transplantation with different graft types according to the primary hepatic circulation of the recipients are discussed. Finally, the flowchart applied in our center for performing graft inflow modulation according to systemic and hepatic hemodynamic parameters during liver transplantation is proposed in detail.

Feng AC, Fan HL, Chen TW, Hsieh CB. Hepatic hemodynamic changes during liver transplantation: A review. *World J Gastroenterol* 2014; 20(32): 11131-11141 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11131.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11131

INTRODUCTION

Liver transplantation (LT) is the optimal treatment for patients with advanced-stage liver disease. However, the systemic and hepatic hemodynamic changes differ from patient to patient, such as the presence of collateral circulation, splenomegaly, or portal vein thrombus. Furthermore, the associated clinical parameters of portal vein flow (PVF), portal vein pressure (PVP), hepatic venous pressure gradient (HVPG), and hepatic artery flow (HAF) might interact, resulting in difficulties in decision making regarding whether to perform graft inflow modulation (GIM). Therefore, strategies aimed at obtaining an optimal blood supply to fulfill the oxygen and metabolic de-

mands of the liver according to each individual represent a critical issue. In this review, we focus exclusively on the hemodynamic changes during LT and the possible correlations between clinically available parameters.

PHYSIOLOGY OF LIVER HEMODYNAMICS

The liver mass constitutes 2.5% of the total body weight or 33 g/kg of body weight[1,2]; however, the liver receives a total blood flow of 100-130 mL/min per 100 g liver and approximately 25% of the cardiac output[3,4]. The liver accounts for 10%-15% of the total blood volume and about 40% of that blood held in large vessels, such as the portal and hepatic veins, with the remaining 60% held in the sinusoids. Half of the hepatic blood can be rapidly expelled from the liver in response to both active and passive influences, thus providing the liver a major role as a blood volume reservoir[5,6].

The hepatic circulation is the most complex system among the organs owing to its dual blood supply from the portal vein and hepatic artery. The hepatic artery normally supplies about 25% of the portal blood flow to the liver, or 30 mL/min per 100 g liver weight (LW), and provides 30%-50% of the liver oxygen requirement with well-oxygenated blood. On the other hand, the portal vein carries about 75% of the total blood flow to the liver, or 90 mL/min per 100 g of liver tissue, and offers approximately 50%-70% of the normal liver oxygen requirement with partially deoxygenated blood of the venous outflow from the entire prehepatic splanchnic vascular bed. In the resting state, the liver accounts for approximately 20% of the total oxygen consumption of the body[7].

The valveless portal vein system is low pressure, low-resistance, and regulated mainly by mesenteric and splanchnic arteriole constriction as well as intrahepatic vascular resistance. The normal PVP is 5-10 mmHg as detected by direct cannulation[8] or the splenic puncture method[9,10], and the pressure in the sinusoid bed or clinical wedge hepatic venous pressure is estimated to be higher than that of the vena cava but slightly less than that of portal vein, with values of 3-10 mmHg[7,11-15]. In contrast, the hepatic artery is a high-pressure, high-resistance system regulated intrinsically by classical arterial autoregulation with a mean pressure similar to that of the aorta[15]. The normal hepatic artery and portal vein hemodynamic supply fluctuate and compensate for each other according to the physiological condition. The well-documented interaction between the hepatic artery and portal vein is termed the hepatic arterial buffer response (HABR)[16], which involves an increase of HAF to compensate for the reduced PVF to minimize the influence of PVF changes on hepatic clearance and to maintain the overall liver blood flow and adequate oxygen supply to tissues[17-20].

However, the PVF and PVP are less strongly correlated. In the normal liver, the PVP is relatively stable even when the PVF fluctuates. The sinusoidal structure and intrahepatic vasculature comprise a compliant vascular bed that can enlarge its volume to accommodate additional portal blood flow without changes in pressure[21-25].

Normally, hepatic hemodynamics change according to the encountered physiological condition and maintain the balance between systems and physical demands; however, this homeostasis is markedly altered in liver diseases and hepatic surgeries such as LT.

HEPATIC HEMODYNAMICS IN CIRRHOSIS-RELATED PORTAL HYPERTENSION

Portal hypertension is defined as a sustained increase in the intraluminal pressure of the portal vein and its collaterals with a mean pressure greater than 12 mmHg, the upper limit for variceal bleeding and other clinical consequences[26]. Cirrhosis-related portal hypertension may result from initial hepatocyte injury and inflammatory necrosis; then, activated stellate cells transform into contractile, fibrogenic myofibroblasts, which produce a large amount of extracellular matrix and inflammatory cytokines, and finally excessive fibrosis[21,27]. The increased sinusoidal resistance and structural changes of the sinusoidal endothelia result in diminished PVF and a reactive increase in PVP[28-31]. In contrast, the splanchnic vasculature undergoes progressive vasodilatation due to excess of vasodilators such as nitric oxide, which is related to increased vascular sheer stress and intestinal absorption of lipopolysaccharide[22,32,33]. Subsequently, vasodilators induce progressive vasodilatation of splanchnic circulation and a related PVF increase along with the development of systemic hyperdynamic circulation with reactive splenomegaly and portosystemic collateralization in multiple locations[34-37]. However, the progressive development of the collateral network and splenomegaly vary individually; it is thought that the development of collateral circulation was due to the opening of pre-existing vascular channels in response to increased PVP[37]. In cirrhotic patients, extrahepatic shunts may account for at least 50% of the portal flow, whereas 80% of the portal flow actually reaching the liver has been observed to bypass the sinusoidal vascular bed *via* intrahepatic shunts[7]. The azygos blood flow has been measured using a double thermodilution catheter directed under fluoroscopy in patients with alcoholic cirrhosis. The azygos blood flow was 596 ± 78 mL/min and 305 ± 29 mL/min in patients with repeated gastroesophageal variceal bleeding and others who underwent decompressive surgery of the portal system[38].

HEMODYNAMIC CHANGES DURING LIVER TRANSPLANTATION

The hemodynamics vary widely among cirrhotic patients who undergo LT owing to different liver disease stages,

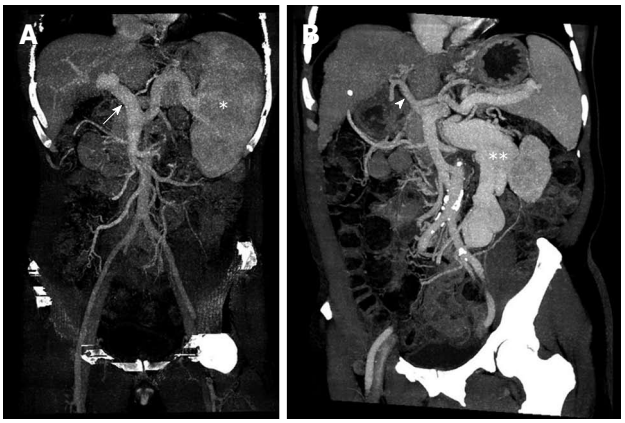

Figure 1 Hepatic and splanchnic vasculature at the time of transplantation in cirrhotic patients. A: Portal vein engorgement (white arrow) and splenomegaly (white astrocyte) without remarkable collateral formation; B: Shrinkage of portal vein (white arrowhead) with massive collateralization (white double astrocytes).

underlying conditions, and disease nature; therefore, it is quite challenging for transplant surgeons to apply individualized transplant strategies. Cirrhotic patients exhibit different hepatic and splanchnic vasculature presentations at the time of transplantation. These are divided into two major types: (1) portal vein engorgement and splenomegaly without remarkable collateral formation; and (2) shrinkage of the portal vein with massive collateralization (Figure 1). The surgical strategies utilized during LT differ not only according to the vasculature but also depending on the graft type, such as a partial or full-sized graft. Thus, the hemodynamic changes during LT are important parameters for determining the optimal surgical strategy.

LIVER TRANSPLANTATION WITH FULL-SIZED GRAFT

Adequate blood supply to the transplanted graft is essential to graft survival and function. In full-sized grafts, it was assumed that the PVF should be at least 1000 mL/min to maintain appropriate organ perfusion[39-41]. However, there are two major types of splanchnic vascular structures observed in patients before LT: (1) portal vein engorgement with splenomegaly but without massive collateral network formation; and (2) shrinkage of the portal vein with remarkable collateralization.

In the first situation, the possibility of developing small-for-size syndrome (SFSS) is less likely because of transplantation with a full-sized graft; however, the HABR becomes an important issue. Pratschke *et al*[42] reported that a decreased PVF of ≤ 1300 mL/min was associated with significantly lower organ survival in univariate analysis, but not in multivariate analysis; in contrast, diminished HAF was associated with an increased rate of primary nonfunction and impaired survival. In the same study, HAF was independent of other confounders with a hazard ratio of 2.5 for poor outcomes, and the clinical cutoff points of 100 and 240 mL/min were identified[42]. In another study, Spitzer *et al*[43] concluded that a target level of PVF > 1 L/min is required in LT to obtain better patient survival; in the hepatic artery, a baseline flow > 250 mL/min is a minimally acceptable level, but > 400 mL/min is ideal[43]. Therefore, when these patients undergo LT with full-sized grafts, two different types of GIM is recommended: splenic artery ligation (SAL) and splenectomy. We recommended that if the PVF is in the range of 1000-1300 mL/min and the HAF is < 100 mL/min, SAL may be the better choice. On the other hand, if the PVF is > 1300 mL/min and the HAF is far < 100 mL/min, splenectomy is likely the best choice if other possible structures or technical conditions are excluded. By performing SAL or splenectomy, excessive PVF can be prevented, which may impair HAF *via* the HABR.

In the second situation involving transplant patients with shrinkage of the portal vein and remarkable collateralization, including portal systemic shunting, the primary issue becomes the need for collateral or shunt ligation. Castillo-Suescun *et al*[44] presented a series of patients diagnosed with spontaneous splenorenal shunts (SSRSs) undergoing orthotopic LT, and shunt disconnection was performed when the post-reperfusion PVF was ≤ 1200 mL/min without any detrimental effects on renal function. However, strong clinical evidence is lacking regarding the performance of collateral or portosystemic shunt disconnection. Because the portal vein normally carries approximately 90 mL/min per 100 g LW[7], ligation of the major collateral vessels or shunts is reasonable and required if the portal perfusion is < 1000 mL/min, as the PVF would be shunted away from the new liver by old collaterals. Margarit *et al*[45] reported two patients underwent occlusion of distal splenorenal shunt during LT with an increase of PVF similar to that of splenorenal shunts. In addition, Esquivel *et al*[46] reported consistent anatomic changes in the portal vein diameter according to the presence of portosystemic shunt of 1.2-1.5 cm, which was smaller compared with other adult recipients without remarkable shunting or collaterals of an average of 2.5 cm[46,47]. Therefore, it is worth noting that when the portal vein diameter is smaller preoperatively by computed tomography or intraoperatively even without obvious collateral shunting, the possibility of portal hypoperfusion should always be taken into consideration.

LIVER TRANSPLANTATION WITH PARTIAL GRAFT

The hemodynamic changes are even more complicated when LT with partial graft is performed because of the higher risk of SFSS. The most appropriate hemodynamic parameter in deciding the application of GIM remains a topic of debate. PVP < 15 mmHg was reported as a key factor for successful adult living donor liver transplanta-

tion (LDLT) with better two-year survival[48]. In a subsequent study conducted by the same group, the cutoff point for intentional PVP modulation was 20 mmHg, which was mainly achieved by splenectomy or additional creation of a portosystemic shunt. Finally, the authors also concluded that intentional PVP modulation at < 15 mmHg is an effective surgical strategy for small-for-size grafts that establishes greater donor safety with good LDLT results[49]. Ito *et al*[50] reported that an elevated PVP of > 20 mmHg early in the first week post-transplantation is strongly associated with an increased incidence of bacteremia in the first three months and worse patient (graft) survival at six months. Other groups use PVP as an indicator for performing GIM, with the acceptable range of 15-20 mmHg[51,52]. The PVF is another parameter utilized by many groups; however, the proper range differs widely according to graft type. Sainz-Barriga *et al*[53] reported that the optimal threshold of four times the flow rate observed in healthy donors (360 mL/min per 100 g LW) is a risk factor for graft failure, and flow rates below the target of 180 mL/min per 100 g LW led to lower survival rates. This observation was confirmed by Hessheimer *et al*[54] in an experimental model. Shimamura *et al*[55] proposed that to avoid SFSS, a PVF of < 260 mL/min per 100 g LW is indicated. Troisi *et al*[56] also reported that a PVF value of 250 mL/min per 100 g LW predicted SFSS development.

In transplantation with partial grafts, HABR plays a crucial role despite the lack of consensus on the optimal range of HAF in LDLT. Sainz-Barriga *et al*[53] published a detailed report on the systemic and hepatic hemodynamics during LT. There was a significant difference in the median HAF between full-sized and partial grafts; however, no significant difference was found when the median HAF was indexed by graft weight. On the other hand, the ratio of PVF to HAF was elevated from the median of 6.6 to 15.4, which represented the effect of HABR[53]. Although no available data focus on the effect of the PVF to HAF ratio, it remains a potential predictor of surgical outcomes[57,58].

Similar to full-sized grafts, there are two major splanchnic vascular structures in patients before LT similarly as mentioned in the previous section. When the patients with engorged portal veins and splenomegaly undergo transplantation with partial grafts, the primary issue is the occurrence of SFSS. In the early era of LDLT, a graft versus recipient weight ratio (GRWR) of < 0.8 or a graft size of < 35% of the estimated standard graft weight were considered major risk factors for SFSS development[59,60]; however, with the evolution of surgical techniques and accumulation of clinical experience, the following investigations showed that the reduction of the lower limit of the GRWR to 0.6 in LDLT is safe[61-64]. The primary strategy employed to achieve better graft function and survival is strict inflow control including PVF and PVP instead of a smaller graft size. Asencio *et al*[65] hypothesized that the development of SFSS is not exclusively determined by the graft size, but instead by the hemodynamic parameters of the hepatic circulation, which indicates that hepatic hyperperfusion is a critical factor. Therefore, when cirrhotic patients with portal vein engorgement and splenomegaly undergo transplants with partial grafts, GIM is required in the majority owing to the potential risk of portal hyperperfusion. However, there is a lack of consensus about the clinical utility of using hemodynamic parameters to determine the need for and the type of GIM. It has been reported that splenic artery occlusion and coronary vein ligation can reduce the portal inflow by 52%[66], and SAL can reduce the portal inflow with a compensatory increase in HAF due to the HABR[67,68]. Troisi and de Hemptinne[56] reported that SAL resulted in a significant decrease in the PVF from 2600 ± 832 to 1700 ± 689 mL/min and a compensatory increase in the HAF from 87 ± 39 to 152 ± 64 mL; thus, SAL represents a simple and safe method that is sufficient to allow portal inflow modulation in most patients[69]. It is believed that intraoperative ligation of the splenic artery causes less than 50% of infarctions[70]. Furthermore, in cirrhotic patients with portal hypertension, splenic artery occlusion caused a significant reduction in PVP from the baseline of 21.5 ± 3.8 to 17.6 ± 3.2 mmHg 15 min after splenic artery occlusion ($P < 0.0001$) in the study conducted by Luca *et al*[71], Ito *et al*[50] reported similar results of immediate reduction of PVP with a median of 16-11 mmHg ($P = 0.02$) after SAL. Splenectomy or SAL is beneficial for improving outcomes of LDLT using a relatively smaller left lobe graft; however, splenectomy remains a life-threatening factor and is technically advanced compared with SAL[72,73]. However, Ikegami *et al*[74] began performing aggressive splenectomy using a vessel-sealing system to control PVP exceeding 20 mmHg, and a better graft survival rate was achieved compared to patients without strict portal pressure control (81.8% *vs* 90.6%, $P < 0.01$); in the same study, the complication rate of splenectomy was 10.1% (9/89), including pancreas leakage and overwhelming postsplenectomy sepsis[74]. Because of its technical simplicity and fewer postoperative complications, SAL can be considered the first-line GIM for portal hyperperfusion followed by subsequent splenectomy for more aggressive control[75].

Flowcharts for GIM have been proposed by different groups primarily based on PVF and PVP; the Kyoto group uses PVP ⩾ 20 mmHg as the cutoff point for performing splenectomy[49]. Asencio *et al*[65] proposed that either PVP > 20 mmHg or PVF > 250 mL/min per 100 g LWis indicated for implementing PVP control maneuvers. In a more detailed algorithm, the Italy group considers a PVF value greater than four times the normal baseline value (⩾ 360 mL/min per 100 g LW) as the first-line determinant for performing portocaval shunt (PCS) or SAL, and then HVPG [HVPG = PVP - central venous pressure (CVP)] ⩾ 15 mmHg is taken into consideration for possible PCS or SAL if the PVF does not exceed 4 times the normal baseline value. Finally, SAL is recom-

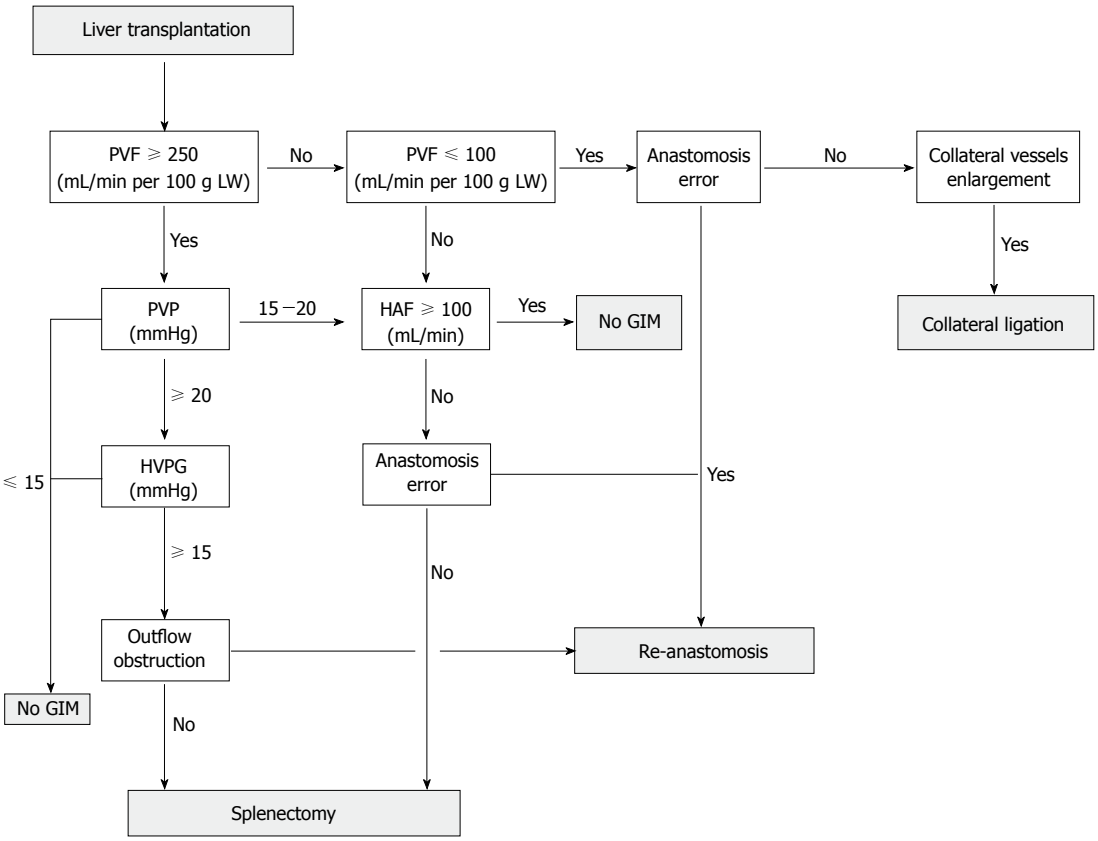

Figure 2 Flowchart applied in our center for performing graft inflow modulation according to systemic and hepatic hemodynamic parameters in transplant patients with portal hyperperfusion. Technical errors such as hepatic outflow obstruction, hepatic artery kinking, or anastomosis failure should be evaluated repeatedly. After graft inflow modulation (GIM) is performed, the portal vein flow (PVF), portal vein pressure (PVP), and central venous pressure should be re-measured to ensure optimal portal inflow. HAF: Hepatic artery flow; HVPG: Hepatic venous pressure gradient; LW: Liver weight.

mended if an HAF > 100 mL/min can be achieved after splenic artery isolation and clump testing under a normal gradient; otherwise, there is no need for further GIM, and the use of heparin and prostacyclin is considered[53]. After summarizing the flowcharts and recommended ranges for all clinically available parameters, the algorithm currently applied in our center for GIM is primarily based on the sequence of PVF, PVP, HVPG, hepatic outflow, and finally HAF (Figure 2). Hepatic outflow obstruction may aggravate the injury caused by excessive inflow with graft congestion, and it is essential to exclude hepatic outflow obstruction as well. Currently, clinical judgment of performing GIM is mainly based on the coloration, induration, and bile production of the graft at reperfusion along with objective parameters such as PVF, PVP, or HVPG, which varies in different centers. In the algorithm applied in our center, we strongly recommend measuring all available hemodynamic parameters required for delicate and individualized surgical strategies, particularly in transplant patients at high risk for developing SFSS.

In the second situation of transplant patients with shrinkage of portal vein and remarkable collateralization receiving partial grafts, the possibility of developing SFSS is lower; however, measurement of all hemodynamic parameters remains essential for determining the need for collateral ligation. Furthermore, portal hypoperfusion can occur when the collaterals are well developed, such as SSRS or coronary veins, and this is particularly common in recipients with portal vein thrombus, an important warning sign[70]. Sainz-Barriga *et al*[53] reported that flow rates below the target of 180 mL/min per 100 g LW led to lower survival rate; therefore, PVF is a major issue owing to the sinusoidal structure of the liver and its reservoir nature related to decreased PVP fluctuation if the veins are not fully distended[53,76-80]. Collateral shunting is a potential risk factor for portal hypoperfusion, and the need for disconnection remains controversial. In this circumstance, if the clinical decision is based solely the coloration and induration of the liver graft at reperfusion, the hypoperfusion state will be judged erroneously. It is worth noting that PVF measurement is required if the preoperative computed tomographic evaluations of the recipient shows a smaller portal vein caliber or the presence of remarkable collateral vessels. If the PVF is < 100 mL/min per 100 g LW, collateral ligation is definitely required, and if the PVF is between 100 to 180 mL/min per 100 g LW, collateral vessel isolation and clump testing should be performed. If the PVF exceeds 260 mL/min per 100 g LW, collateral ligation might not be needed in order to prevent portal hyperperfusion and decrease the HAF due to excessive HABR.

In order to describe the concept and rationale for

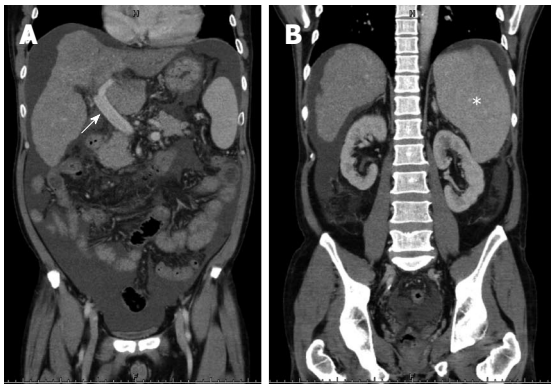

Figure 3 Preoperative computed tomographic findings of case 1. A: Engorged portal vein of 1.8 cm in diameter (white arrow); B: Splenomegaly with 15.4 cm at the longest axis without remarkable collaterals (white asterisk).

GIM according to various hemodynamic parameters, we next describe two cases of LT performed in our hospital to illustrate how optimal graft inflow can be achieved.

CASE 1

A 49-year-old man weighing 69 kg and diagnosed with hepatitis B-related liver cirrhosis, Child-Pugh B, and a hepatocellular carcinoma of approximately 6 cm in segment four, received a 600-g right lobe liver graft from his daughter. Preoperative computed tomography showed that the portal vein was 1.8 cm in diameter and splenomegaly was present with 15.4 cm at the longest axis without remarkable collateral vessels (Figure 3A and B). After portal vein isolation, the PVF before transplantation was 972 mL/min (162.0 mL/min per 100 g LW) measured by a transonic flowmeter with an ultrasonic probe encircling the main portal vein. The PVP was 31 mmHg detected by the direct puncture method and the CVP level was 11 mmHg. On reperfusion, the portal inflow was 1936 mL/min (322.7 mL/min per 100 g LW), the HAF was 93 mL/min, the PVP was 20 mmHg, and the CVP level was 7 mmHg. Considering the risk of SFSS development, SAL was performed first, and the PVF decreased to 1862 mL/min (310.3 mL/min per 100 g LW), the HAF was 94 mL/min, the PVP was 18 mmHg, and the CVP level was 8 mmHg. The PVF remained > 260 mL/min per 100 g LW; therefore, splenectomy was performed. After splenectomy, the PVF decreased to 1385 mL/min (230.8 mL/min per 100 g LW), the HAF increased to 115 mL/min, the PVP was 15 mmHg, and the CVP remained at 8 mmHg with an HVPG of 7 mmHg. The postoperative course was uneventful, and the patient was discharged from the hospital eight days after the operation (Figure 4).

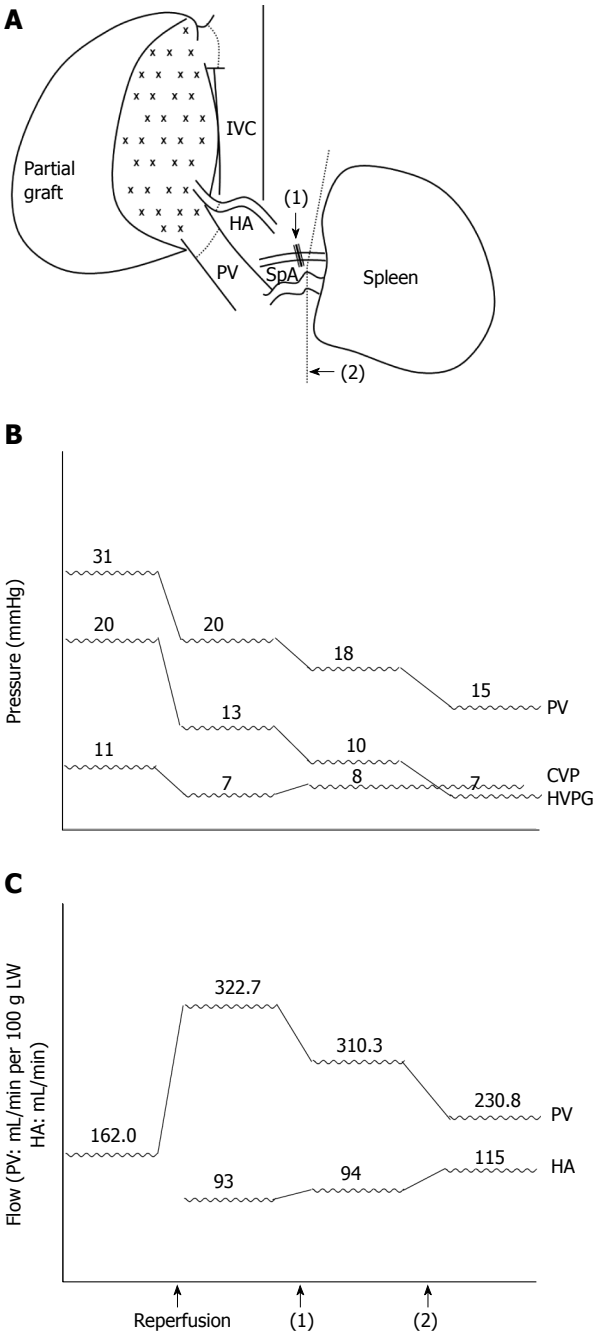

Figure 4 Sequential graft inflow modulation of case 1. A: Splenic artery ligation [black arrow, (1)] followed with splenectomy [black arrow head, (2)]; B: Sequential changes of the portal vein (PV) pressure, central venous pressure (CVP), and hepatic venous pressure gradient (HVPG) during liver transplantation pre- and post-GIM; C: Sequential changes of the portal vein flow (mL/min per 100 g of LW) and hepatic artery (HA) flow (mL/min). IVC: Inferior vena cava; SpA: Splenic artery.

CASE 2

A 55-year-old man weighing 89 kg with hepatitis B-related liver cirrhosis complicated by bleeding esophageal varices and a 4-cm hepatocellular carcinoma in segments six and seven underwent LDLT with a 620-g right lobe graft from his son. Preoperative computed tomography showed that the right lobe portal vein could not be identified, likely due to hepatofugal flow-induced stasis and thrombosis; splenomegaly (long axis measuring about 12.5 cm), ascites, esophageal varices, a portal-systemic shunt from the left portal vein to the left pericardiac-

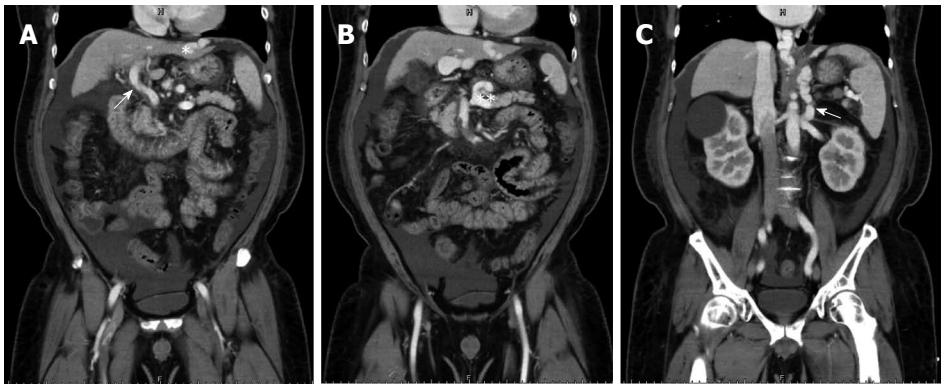

Figure 5 Preoperative computed tomographic findings of case 2. A: The right lobe portal vein could not be identified, likely owing to hepatofugal flow-induced stasis and thrombosis (white arrowhead) and portal-systemic shunting from the left portal vein to the left pericardiac-phrenic vein (white asterisk); B: Coronary vein engorgement (double white asterisks); C: Spontaneous splenorenal shunting was also noted (white arrow).

phrenic vein, and SSRS were also noted (Figure 5). Before transplantation, the PVF was 136.0 mL/min (21.9 mL/min per 100 g LW), the PVP was 28 mmHg, and the CVP was 12 mmHg. At reperfusion, the PVF was 240 mL/min (38.7 mL/min per 100 g LW). Therefore, we performed hepatic artery anastomosis immediately with flow rate of 90 mL/min, and subsequent coronary vein ligation without measuring PVP because of portal hypoperfusion. Finally, the PVF was 908 mL/min (146.5 mL/min per 100 g LW), the HAF was 73.5 mL/min, the PVP was 18 mmHg, the CVP was 13 mmHg, and the HVPG was 5 mmHg. The patient recovered well after the operation and was discharged 10 d later (Figure 6).

DISCUSSION

These two cases focused primarily on the two most frequently encountered situations for transplant surgeons: portal hyperperfusion and hypoperfusion. In the first case, the preoperative evaluation showed an engorged portal vein with splenomegaly, which were both considered the presentation of severe portal hypertension without well-developed collaterals, thus GIM might be required even if the GRWR was more than 0.8. We had performed a series of measurements of PVF, PVP, HAF, and CVP; the GIM of SAL and splenectomy were considered sequentially with the consideration of all hemodynamic parameters instead of PVF or PVP alone. Finally, optimal PVF, PVP, and HVPG values were achieved without difficulty. There is always a debate about the best indicator for GIM, such as PVF, PVP, or even HVPG; however, our group takes all available parameters into account and proposes an individualized transplant procedure instead.

In the second case, preoperative computed tomography revealed the presence of possible portal vein thrombus and abundant portosystemic collaterals with portal vein shrinkage, which increased the risk of the development of portal hypoperfusion after transplantation even with a smaller partial graft (GRWR < 0.8). After reperfusion, PVF measurement was performed routinely despite subjective assessment of the graft being soft without congestion, and portal hypoperfusion was noted immediately. Therefore, hepatic artery anastomosis was performed without delay with subsequent ligation of the coronary vein, which was easier and less risky than splenorenal shunt ligation. Finally, satisfying results were also obtained without surgical complications.

We strongly recommend that all hemodynamic parameters should be monitored during surgery to evaluate the graft status instead of clinical subjective observations of the graft consistency and coloration. Additionally, transplant surgeons should review the preoperative studies carefully to evaluate the possibility of portal hypoperfusion or hyperperfusion instead of considering GRWR only.

CONCLUSION

The delicate control of all hemodynamic parameters during LT is critical for better graft survival and a reduced risk of perioperative complications. Although a lack of consensus remains regarding the best clinical parameter such as PVF or PVP, measurement of all available hemodynamic indices is essential for developing an individualized surgical plan not based on clinical judgment alone. Preoperative evaluation of the present portal and splanchnic vasculature is warranted; in addition to graft type, surgeons can predict the likelihood of developing portal hyperperfusion or hypoperfusion states, which may lead to poor surgical outcomes. For transplant surgeons, the most challenging aspect of LT is not only the complex surgical techniques used but also the modulation of portal inflow during the operation that require comprehensive consideration of all hepatic and systemic hemodynamics both anatomically and clinically. Presently, there is a lack of statistical correlation between PVF and PVP; therefore, there is no consensus about the clinical utility of these parameters to serve as a practical indicator for performing graft inflow modulations. Further investiga-

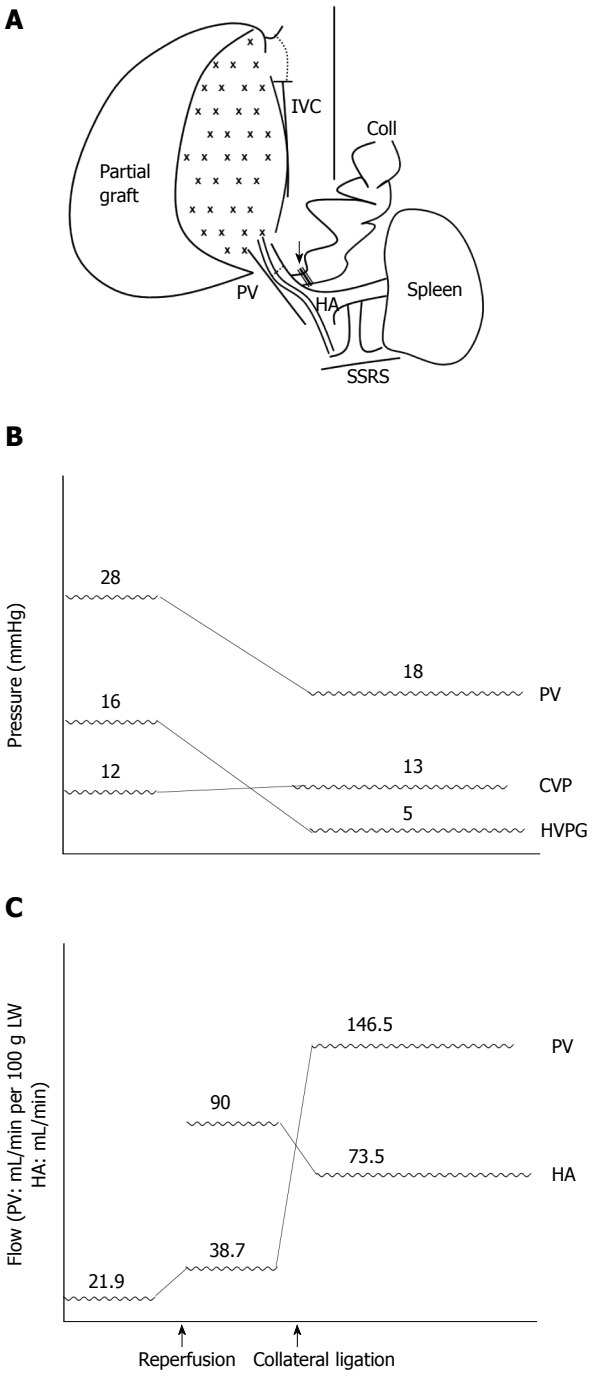

Figure 6 Sequential graft inflow modulation of case 2. A: After transplantation with a right-lobe graft, hypoperfusion was noted and coronary vein ligation was performed immediately (black arrow); B: Sequential changes of the portal vein (PV) pressure, central venous pressure (CVP), and hepatic venous pressure gradient (HVPG) during liver transplantation pre- and post-coronary vein ligation; C: Sequential changes of the portal vein flow (mL/min per 100 g of LW) and hepatic artery flow (mL/min). Coll: Collateral; HA: Hepatic artery; IVC: Inferior vena cava; SSRS: Spontaneous splenorenal shunt.

tion is required to identify a more generalized parameter derived from not only the hepatic but also the systemic hemodynamic status, which will represent a more reliable predictor for surgical outcomes and a potential intraoperative determinant of GIM.

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P- Reviewer: Hatipoglu S, Kaido T, Popescu I **S- Editor**: Qi Y
L- Editor: A **E- Editor**: Zhang DN

World Journal of Gastroenterology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i32.11142

World J Gastroenterol 2014 August 28; 20(32): 11142-11159
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (14): Pancreatic cancer

Therapeutic options for the management of pancreatic cancer

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Author contributions: Rossi ML and Gondi CS provided the conception and design the article; Rossi ML, Rehman AA and Gondi CS drafted the article; Rossi ML and Gondi CS revised the critically important content, and provided the final approval of the version to be published.

Supported by Grant from the William E McElroy Foundation, Springfield, IL 62794-9264, United States to Gondi CS; and the Carrie M. Craig Medical Student Summer Research Fellowship Program sponsored by the University of Illinois College of Medicine at Peoria, Box 1649, Peoria, IL 61656-1649, United States to Rossi ML

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Received: October 25, 2013 Revised: January 11, 2014
Accepted: May 28, 2014
Published online: August 28, 2014

Abstract

Since its initial characterization, pancreatic ductal adenocarcinoma has remained one of the most devastating and difficult cancers to treat. Pancreatic cancer is the fourth leading cause of death in the United States, resulting in an estimated 38460 deaths annually. With few screening tools available to detect this disease at an early stage, 94% of patients will die within five years of diagnosis. Despite decades of research that have led to a better understanding of the molecular and cellular signaling pathways in pancreatic cancer cells, few effective therapies have been developed to target these pathways. Other treatment options have included more sophisticated pancreatic cancer surgeries and combination therapies. While outcomes have improved modestly for these patients, more effective treatments are desperately needed. One of the greatest challenges in the future of treating this malignancy will be to develop therapies that target the tumor microenvironment and surrounding pancreatic cancer stem cells in addition to pancreatic cancer cells. Recent advances in targeting pancreatic stellate cells and the stroma have encouraged researchers to shift their focus to the role of desmoplasia in pancreatic cancer pathobiology in the hopes of developing newer-generation therapies. By combining novel agents with current cytotoxic chemotherapies and radiation therapy and personalizing them to each patient based on specific biomarkers, the goal of prolonging a patient's life could be achieved. Here we review the most effective therapies that have been used for the treatment of pancreatic cancer and discuss the future potential of therapeutic options.

Key words: Pancreatic cancer; Pancreatic cancer stem cells; Microenvironment; Surgical resection; Neoadjuvant therapy; Adjuvant therapy; Chemotherapy; Radiation; Personalized therapy

Core tip: Pancreatic ductal adenocarcinoma has challenged researchers for decades. It remains one of the most deadly cancers due to the complex molecular and genetic makeup of its cancer cells and their surrounding microenvironment. In addition, there are no valid screening tests available to detect pancreatic cancer in its early stages. Yet, as knowledge of this cancer has evolved over time, so have novel methods for treating it. Researchers have a deeper understanding of pancreatic cancer now than ever before. The future holds much promise for new breakthroughs that will significantly improve patient outcomes.

Rossi ML, Rehman AA, Gondi CS. Therapeutic options for the management of pancreatic cancer. *World J Gastroenterol* 2014;

20(32): 11142-11159 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11142.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11142

INTRODUCTION

Despite the improved survival rates noted in numerous cancers, including breast[1-3], prostate[4] and colon cancer[5], the overall survival rates for patients diagnosed with pancreatic cancer have shown little improvement over the past thirty years[6-8]. Pancreatic ductal adenocarcinoma (PDA) remains one of the most rapidly progressive and deadly malignancies worldwide[4]. The prevention of pancreatic cancer is difficult to assess, due to limited studies identifying potential risk factors compounded with the multifactorial, heterogeneous nature of the disease. Cigarette smoking has been noted to double the risk of pancreatic cancer, yet only accounts for 20%-25% of the cases[9,10]. Additionally, family history may also contribute a significant role as 5%-10% of individuals with pancreatic cancer report an incidence of pancreatic cancer in a close family member[11]. This risk is further substantiated when there is a larger number of family members with pancreatic cancer and a decrease in age of onset in kindred[12]. Other noted risk factors include alcohol abuse[13], a high-fat diet[14,15], and certain trace elements[16].

The challenge of diagnosing PDA at an early stage further contributes to the dismal five-year survival rate that is projected for patients. Located in the retroperitoneum of patients who present with non-specific symptoms, PDA is not diagnosed until it has reached an advanced clinical stage in 80% of patients[17]. In addition, lack of effective screening and early biomarker detection methods have prevented clinicians from identifying this cancer in a pre-malignant stage. Ideally, visual evaluation *via* computerized tomography (CT) and magnetic resonance imaging (MRI) should be incorporated upon suspicion of pancreatic cancer for detection and resectability assessment[18]. Although CT scan has often been utilized to detect pancreatic cancer[19-21], reliance on MRI, particularly in regard to assessing local invasion and metastasis, has increased[22]. Other imaging may also provide certain benefits, such as endoscopic ultrasound for investigating vascular invasion[23], fludeoxyglucose-positron emission tomography scanning for recurrent tumors[24], and laparoscopy for more accurate staging[25]. While the use of these techniques remains helpful to determine prognosis and treatment regimen for patients diagnosed with pancreatic cancer, none have been validated as effective screening tests for general or high risk populations.

Once diagnosed, a number of chemotherapy, radiation and combination therapy regimens have been used to treat patients with ductal pancreatic tumors. Unfortunately, the dynamic molecular and cellular makeup of individual pancreatic tumors, renders many of them resistant to the majority of these therapies. Although surgical resection has been shown to increase patient survival by 10 mo[26], the majority of patients who undergo these procedures experience comorbidities and recurrence. Current research has identified additional sources of therapeutic resistance in the microenvironment of these tumors. Characterized by stromal proliferation, reduced angiogenesis and a unique subset of cells known as cancer stem cells (CSCs), the tumor microenvironment has become a target of new therapeutic agents.

While improved understanding of pancreatic cancer biology has lead to several therapeutic breakthroughs in the treatment of PDA, major progress toward improving survival rates in patients has been extremely slow. However, as our understanding of this tumor's therapeutic resistant nature improves, so will future progress in treating pancreatic cancer.

CLINICAL PRESENTATION AND DIAGNOSIS

One of the greatest challenges in treating pancreatic ductal adenocarcinoma (PDA) is discovering it in the pre-malignant stage. The average patient diagnosed with pancreatic cancer is in their seventh decade of life and presents to their primary care physician with general symptoms of abdominal pain and weight loss[27]. Not only is the pancreas difficult to palpate due to its retroperitoneal location, but there are also no specific blood tests to confirm suspicion of malignancy. More specific symptoms, such as unexplained jaundice[28], onset of diabetes[29] and development of thromboembolic disease[30] are more diagnostic of pancreatic cancer, but do not present until later stages of the disease. The primary comorbidities associated with PDA include biliary obstruction, infection, ascites, pancreatic insufficiency and in advanced stages of the disease, cachexia[31]. Unfortunately, once a patient presents with these symptoms, the disease has often already reached its malignant stage and the patient may never be able to receive treatment.

Effective screening tests to provide early diagnosis of pancreatic cancer could potentially prevent these symptoms. The ones that do exist are not validated. For example, although endoscopic ultrasounds provide a higher yield of detecting pancreatic cancer in its early stages, the comorbidities associated with this procedure render it an unsuitable screening test in the general population. As a result, studies are currently underway to identify high risk individuals who may benefit from this invasive procedure[32-34]. Other techniques, such as cross-sectional imaging could be used to identify asymptomatic pancreatic neoplasms for surgical resection[34] as long as they are confirmed by CT or MRI techniques which provide better resolution between normal and neoplastic pancreatic tissue[35].

Although a greater understanding of the molecular events in pancreatic cancer tumorogenesis has lead to the discovery of biomarkers that help to predict the tumor's response to treatment, there has been no use of these markers in cancer drug development[36]. The only biomarker

Table 1 Cellular mechanisms of therapeutic resistance in pancreatic cancer

Cellular pathways	Mutated gene	Ref.
Cell-cycle control	*CDK2NA* (90%); *APC2*	Almoguera *et al*[54]; Schutte *et al*[71]; Hahn *et al*[72]
RAS	*KRAS* (90%); *MAP2K4*	Almoguera *et al*[54]; Hruban *et al*[56]; Pellegata *et al*[57]; Hezel *et al*[58]; Maitra *et al*[59]
DNA damage repair	*TP53* (75%-90%)	Almoguera *et al*[54]; Redston *et al*[67]; Olive *et al*[68]
TGF-β	*DPC4* (50%), *SMAD4*	Almoguera *et al*[54]; Yachida *et al*[73]
Apoptosis	*CASP10*; *CAD*	Jones *et al*[77]
Cell adhesion	*FAT*; *PCDH9*	Jones *et al*[77]
Hedgehog	*GLI1*; *GLI3*	Jones *et al*[77]
Integrin	*ILK*; *LAMA1*	Jones *et al*[77]
JNK	*MAP4K3*; *TNF*	Jones *et al*[77]
Small GTPases	*PLCB3*; *RP1*	Jones *et al*[77]
Wnt-β-catenin	*MYC*; *TSC2*	Jones *et al*[77]

APC: Adenomatous polyposis coli; CDK2NA: Cyclin-dependent kinase inhibitor 2 A; CAD: Carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase; CASP10: Caspase 10; DPC4: deleted in pancreatic cancer, locus 4; FAT: Fat tumor suppressor; GLI 1: Glioma-associated oncogene; GLI3: Glioma-associated oncogene 3; GTPases: Guanosine triphosphate; Wnt-B-catenin: Wingless type B-catenin; ILK: Integrin-linked kinase; JNK: c-Jun N-ternmial kinases; KRAS: Kristen rat sarcoma; LAMA1: Laminin A-1 chain; MAP2K4: Mitogen-activated protein kinase kinase 4; MAP4K3: Mitogen-activated protein-3 kinase-3: TNF: Tumor necrosis factor; MYC: Myelocytomatosis oncogene; PCDH9: Procadherin 9; PLCB3: Phospholipase C, beta 3; RP1: Retinitis pigementosa 1; SMAD4: Mothers against decapentaplegic homolog 4; TGF-β: Transforming growth factor β; TP53: Tumor protein 53; TSC2: Tuberous sclerosis 2; RAS: Rat sarcoma.

that has shown a great deal of promise in therapeutic monitoring and in identifying the recurrence of pancreatic cancer after treatment is the carbohydrate antigen 19-9 (CA 19-9), a sialylated Lewis blood group A antigen secreted by many pancreatic lesions[37]. Yet, CA 19-9 is not specific for pancreatic cancer and therefore cannot be used to screen for this tumor. Several other conditions, including hepatobiliary diseases, pancreatic diseases and gastrointestinal malignancies, bronchitis, congestive heart failure, cystic fibrosis, diverticulitis, lung cancer, ovarian cysts, pleural effusions, renal cysts and rheumatoid arthritis may present with elevated levels of CA 19-9[38]. In addition, approximately 10% of patients with pancreatic cancer are negative for Lewis antigen a or b. As a result, these patients are unable to synthesize CA 19-9 and will have no detectable levels of this biomarker, even in advanced stages of pancreatic cancer[39]. Although measurement of serum CA 19-9 levels has clinical significance in determining treatment and prognosis for patients with known pancreatic cancer, its usefulness as a diagnostic screening tool of the disease is not substantiated[40].

Upon diagnosis of pancreatic cancer, treatment and management of patients should utilize a multidisciplinary team including, primary care physicians, medical oncologists, radiation oncologists, surgeons, endocrinologists, radiologists and pathologists[41]. Cancer staging subsequently follows, with the American Joint Committee on Cancer providing the standard model, based upon the tumor-node-metastasis system[42]. However, not all criteria regarding tumor staging can be measured prior to surgical intervention. As a result, the majority of staging for pancreatic cancer incorporates imaging results and liver function tests. From these results, patients with pancreatic cancer can often be classified into three major cohorts: (1) patients with a resectable tumor or borderline resectable tumor; (2) patients with a locally invasive tumor without metastasis; and (3) patients with a systemically disseminated tumor.

Appropriately designating cases into the proper subgroup is vital to ensure appropriate treatment and management of patients presenting with pancreatic cancer. Often, fine needle aspiration guided by endoscopic ultrasonography is necessary to obtain histological confirmation[43,44], especially prior to the initiation of chemotherapy and radiation. Throughout the treatment process, CA 19-9 should be continuously measured[45,46]. Nonetheless, previous studies still support the usefulness of CA 19-9 in predicting patient response to chemotherapy[47,48], preoperative prognosis[49], as well as assessing treatment response[50,51], overall survival[51-53], and recurrence[51].

THERAPEUTIC RESISTANCE IN PANCREATIC CANCER

Cellular mechanisms of therapeutic resistance

In an effort to understand the therapeutic resistant (Table 1) nature of PDA, researchers have attempted to characterize the molecular and cellular components of the pancreatic cancer cells as well as the desmoplasia that surrounds them. Although pancreatic cancer displays pathologic and clinical heterogeneity, data suggests the majority of PDA express a successive accumulation of highly penetrant genetic alterations that occur at four genetic loci: K-ras, p53, cdkn2a and smad4/DPC4[54]. Originating in the ductal epithelium, pancreatic cancer evolves from a premalignant lesion to a highly invasive metastatic disease[55].

Ninety percent of tumors have point mutations that are specific for the KRAS2 oncogene, resulting in the constitutive production of the Ras protein[56-59]. Occurring early in tumorogenesis, these point mutations are essential for maintaining the malignant phenotype because once activated, Ras initiates a signal transduction cascade that activates proliferative and cell survival pathways and increases cell invasion[60,61]. The majority of the point mutations occur on codon 12 of the ras protein and give rise to pancreatic tumor-specific neo-antigens. Several studies demonstrated these antigens are recognized by helper T-cells and cytotoxic T-cells[62,63]. Using this knowledge, scientists developed personalized peptide vaccines corresponding to the K-ras mutations present in the tumors of patients enrolled in one clinical trial[64]. The vaccine was

Table 2 Extracellular mechanisms of thereapeutic resistance in pancreatic cancer

Potential therapeutic targets	Extracellular response	Ref.
K-ras mutant oncogene	Proliferation of desmoplastic reaction (leukocytes, fibroblasts, endothelial cells, neuronal cells, collagen, hyaluron): upregulation of GM-CSF	Chu *et al*[78]; Neesse *et al*[79]; Ying *et al*[81]; Nolan-Stevaux *et al*[82]; Bayne *et al*[87]
Sonic hedgehog (SHH)	Growth and differenatiation of stromal fibroblasts	Bailey *et al*[83]; Tian *et al*[84]; Olive *et al*[85]
Tumor associated macrophages (TAMs); cancer associated fibroblasts (CAFs); regulatory T-cells (Treg); myeloid derived suppressor cells	Evasion of the immune system	Bayne *et al*[87]; Pylayeva-Gupta *et al*[88]
Desmoplastic reaction	Anti-angiogenesis; hypoxic tumor environment	Komar *et al*[86]

K-ras: Kinase-rat sarcoma; GM-CSF: Granulocyte macrophage colon-stimulating factor.

proven safe and tolerable and resulted in a more efficient immunologic attack on the tumor[65]. As a result, patients given the vaccine demonstrated a more favorable clinical outcome than those not given the vaccine. Combined with surgical resection, a long-term immune response initiated by the K-ras vaccine has resulted in a 10-year survival in some patients. These results may implicate a role for the K-ras vaccine as an adjuvant treatment option in the future[66,67].

Rather than being activated like the mutated KRAS2 oncogene, the p53 tumor suppressor gene is inactivated in 75%-90% of pancreatic tumors[68,69]. As a result, there is an impaired response to DNA damage in pancreatic epithelial cells, impaired apoptosis and impaired cell cycle control[70,71]. Two other tumor suppressor genes, *p16Ink4a* and *p15ARF* are encoded by the cdkn2a locus. Inactivation mutations in these genes are present in about 90% of human PDA[72,73].

A fourth common mutation seen in more than half of pancreatic cancers causes an alteration in DPC4[74]. These mutations confer a metastatic phenotype. The genetic makeup of the patient determines the number and combination of these mutations that will be present in their PDA. Patients with three or four mutated genes will have a much worse prognosis than those with one or two. The variable expressivity of these tumors presents a challenge in effectively treating them[75].

In addition to these four primary genetic alterations in PDA many other less-frequent mutations occur as well[76-78]. According to a comprehensive genetic analysis of 24 pancreatic cancers, an average of more than 60 genetic abnormalities, primarily point mutations, per tumor were noted in the PDA phenotype. While these mutations have been organized into 12 functional cancer-relevant pathways, not all tumors have alterations in each of these pathways. In addition, key mutations in select pathways appear to differ from one tumor to another[78]. These pathways may confer therapeutic resistance in the pancreatic tumor. Significant genomic instability in pancreatic cancer may also reduce the effectiveness of therapeutic agents by contributing to acquired chemoresistance.

Extracellular mechanisms of therapeutic resistance

Paracrine signals from pancreatic cancer cells stimulate the extracellular proliferation of leukocytes, fibroblasts, endothelial cells, neuronal cells, collagen and hyaluron (Table 2). This extracellular proliferation of cells is known as a desmoplastic reaction. It forms a thick stromal environment around the pancreatic cancer cells[79,80]. Studies have demonstrated that the signals that influence the proliferation of the desmoplastic reaction originate from the K-ras mutant oncogene in the epithelium of the pancreatic cancer cells[81,82].

In addition to the K-ras mutant signaling pathway, there has been an effort by researchers to understand the roles of other signaling pathways between the pancreatic cancer cells and their microenvironment. Sonic hedgehog (SHH) functions similarly to the K-ras mutant. Although it is over expressed in pancreatic cancer cells during the early stages of their development, SHH does not act on the SHH pathway in these cells[83]. Instead, it acts in a paracrine fashion in the extracellular fibroblasts, resulting in their growth and differentiation[84,85].

The desmoplastic reaction not only provides a mechanical barrier to the pancreatic cancer cells, but it is also thought to contribute to the anti-angiogenic environment that is characteristic of pancreatic ductal adenocarcinoma. Both properties directly affect therapeutic efficacy. Inadequate drug delivery to the site of the tumor is directly correlated with a negative patient outcome[86].

Researchers have also suggested a role for the tumor stroma in the T-lymphocyte depleted microenvironment of the PDA. Several cell types found in the desmoplastic reaction have been associated with tumor associated macrophages, cancer associated fibroblasts, regulatory T-cells and myeloid derived suppressor cells. In addition, a role for a K-ras dependent signaling molecule has been shown to up-regulate granulocyte-macrophage colony stimulating factor when activated. This cytokine promotes the maturation of immature myeloid progenitor cells into myeloid derived suppressor cells[87,88].

TREATMENT OF PANCREATIC CANCER

Surgical resection

Although surgical resection offers hope for curative therapy, only 20% of patients present with potentially resectable tumors[89,90]. It is important to note that surgical resection is only considered in patients with completely

Table 3 Therapies for the management of pancreatic cancer

Therapeutic option	Subset	Ref.
Surgical resection	Cephalic pancreatoduodenectomy	Hidalgo[41]
	Distal pancreatectomy	
	Total pancreatectomy	
Chemotherapy	Neoadjuvant	Lemmens *et al*[101];
	Gemcitabine	Gillen *et al*[102];
	Adjuvant	Neoptolemos *et al*[108];
	Gemcitabine	Burris *et al*[118];
	5-Fluorouracil	Heinemann *et al*[119];
	Advanced disease	Reni *et al*[120];
	Gemcitabine	Moore *et al*[122];
	Gemcitabine + fluropyrimidines	Neesse *et al*[79]
	Gemcitabine + platinum analogs	
	Gemcitabine + erlotinib	
	FOLFIRINOX	
	Nab-paclitaxel	
Radiation therapy	Neoadjuvant	Pisters *et al*[131];
	Radiation + 5-fluorouracil	Hong *et al*[133];
	Radiation + paclitaxel	Yeo *et al*[140];
	Proton beam radiation + capecitabine	Regine *et al*[138];
	Adjuvant	Neoptolemos *et al*[137];
	Radiation + 5-Fluorouracil	Moertel *et al*[144];
	Radiation + gemcitabine	Schellenberg *et al*[147]
	Radiation + chemotherapy	
	Advanced	
	Radiation + 5-fluorouracil	
	Radiation + chemotherapy	
	Stereotactic body radiotherapy	
Personalized therapy	Target specific point mutations	Jones *et al*[77];
	Mitomycin C	Villarroel *et al*[153];
	Immune system stimulation	Yanagimoto *et al*[154]

resectable or borderline-resectable tumors (Table 3). Depending on the size and location of the tumor, three operative procedures are potentially utilized, as noted by Hidalgo[41], with additional removal of adjacent lymph nodes: (1) cephalic pancreatoduodenectomy (whipple procedure); (2) distal pancreatectomy; and (3) total pancreatectomy.

Although additional palliative care is often utilized, controversy surrounds the potential benefits. For example, almost 80% of patients presenting with tumors in the pancreatic head exhibit jaundice due to biliary obstruction[91,92]. However, previous investigations have conflicting results regarding preoperative biliary drainage with certain studies reporting a decrease in perioperative morbidity and mortality[93] while others concluding recognizable benefit[94,95].

Preoperative biliary stenting doubled between 1992 and 2007 due to evidence demonstrating a higher risk of postoperative complications in patients presenting with a tumor in the head of the pancreas. Biliary drainage was further supported by evidence demonstrating its ability to improve liver function, nutritional status and cell-mediated immune function[93]. Despite intentions to reduce post-operative morbidity and mortality by improving liver function, extensive clinical studies have demonstrated preoperative biliary stenting prolongs time to operation, increases preoperative infection and is associated with overall increased complication rates after surgical procedures[94,95]. As a result, most studies have advised against routinely performing preoperative biliary drainage and have recommended that patients presenting with jaundice due to a resectable and non-metastatic tumor in the head of the pancreas should undergo early surgery without preoperative biliary resection[95]. Currently, the only indications for preoperative biliary decompression are for patients who present with severe jaundice, are undergoing neoadjuvant therapy, or have had their surgery postponed due to logistics[94,95].

Several poor predictors for successful resection have been identified, including lymph node involvement[96], high tumor grade[97], large tumor size[98], elevated CA 19-9 levels[46], and positive margins of tumor following resection[41]. These same factors also contribute to recurrence of pancreatic tumors. Even with surgical resection, 5-year survival rates remain dismal, at approximately 20% following surgery[90]. However, perioperative complications and mortality have significantly decreased over the past decade, likely due to greater hospital clinical volume through centralization[99,100].

Chemotherapy

Neoadjuvant therapy: Certain patients might receive neoadjuvant therapy, especially if the tumor presents with borderline resectability. In a study utilizing gemcitabine-based chemotherapy, improved tumor resection with increased survival rates was noted in border-line resect-

able cases[101,102]. However, these effects may only occur in select tumors, with influences by both the genetic composition and microenvironment of the pancreatic cancer[103,104]. For example, White *et al*[105] noted p53 mutations were more common in patients with large residual tumors following treatment with chemoradiation. Moreover, outcomes for neoadjuvant therapy prior to surgically-resectable tumors did not differ when chemotherapy was provided post-operatively[106]. Chemotherapy with radiation has also been shown to improve survival, but not stage, of cases presenting with locally invasive tumors without metastasis[107]. However, previous studies do note that surgical interventions are more challenging and increased postoperative stay is associated with patients undergoing resection after neoadjuvant chemoradiation therapy for locally invasive cancer[106]. Since metastatic pancreatic cancer cannot be completely resected, surgical options are unavailable and hence no neoadjuvant therapy can be provided. Lastly, it is important to note that prior to initiating neoadjuvant therapy, histological confirmation of pancreatic adenocarcinoma is required, unlike surgical resection, which often relies solely on imaging.

Adjuvant chemotherapy: Even following complete, successful resection of pancreatic tumors, overall survival and prognosis remains discouraging. Hence, postoperative chemotherapy or chemoradiation is almost always incorporated in the therapeutic regimen. Postoperative chemotherapy often utilizes gemcitabine or 5-fluorouracil (with concurrent leucovorin as a rescue agent). Both drugs have demonstrated significant increases in patient survival, regardless of initial case presentation. These drugs may also be given simultaneously, however, significant toxicity (especially gastrointestinal) has been reported. Although gemcitabine has often been considered the standard, previous studies do differ on which agents are associated with the most optimal benefits. In a phase Ⅲ, randomized control trial, Neoptolemos *et al*[108] noted no significance difference in survivorship between gemcitabine and 5-fluorouracil (with folinic acid) in patients with resected tumors. In a separate study published in JAMA, the authors concluded that gemcitabine alone should be favored over 5-fluorouracil with leucovorin due to its decreased toxicity[108].

Developments of other complementary agents to enhance chemotherapeutic effects are currently under review. For example, possible inhibition of Hedgehog signaling[84] or concurrent use of Smac mimetics[109], microRNAs[110], Resveratrol[111], capecitabine[112], thymoquinone[113] or heat-shock protein complements[114] may promote tumor uptake and damage of administered drugs, such as Gemcitabine administered with concurrent curcumin may also be a potential option, especially in tumors exhibiting gemcitabine-resistance[115]. In addition to utilizing CA 19-9 and imaging to monitor patient response to chemotherapy, other markers, such as human equilibrative nucleoside transporter 1 levels have also shown to be useful[116]. Other gene expression levels, as noted in Fujita *et al*[117], may also be predictive of treatment efficacy, particularly with gemcitabine. Further investigation is required as to whether adjuvant chemotherapy should be administered if prior neoadjuvant therapy before surgery had already been provided.

Chemotherapy for advanced disease: Due to its poor detection rate, 60%-70% of patients present with metastatic pancreatic cancer upon initial diagnosis. In the advanced stage of disease, pancreatic cancer causes imminent mortality for the majority of affected patients and median survival rate is typically 6-8 mo. Therefore, treatment of patients with metastatic pancreatic adenocarcinoma incorporates chemotherapy, targeted-therapy, comorbid conditions, intensive supportive treatment and psychosocial support.

Gemcitabine is currently considered the chemotherapeutic standard of care in treatment of advanced pancreatic cancer[118]. It has been shown to prolong the average survival rate by 4 mo. In an attempt to improve survival rates, several phase Ⅱ and phase Ⅲ trials combined Gemcitabine with fluoropyrimidines and platinum analogs. Most of these combinations failed to show statistically significant survival benefit, however compared to Gemcitabine alone[119]. In another attempt to prolong patient survival, scientists have developed several Gemcitabine-based polychemotherapy regimens involving 3-4 cytotoxic agents. When Reni *et al*[120] performed a randomized trial to test the cisplatin, epirubicin, fluorouracin and gemcitabine (PEFG-regimen) against gemcitabine alone, those patients treated with the PEFG saw a significant decrease in cancer progression, when compared to those treated with gemcitabine alone[120]. Yet in regards to survival, the infusional 5-FU/folinic acid, irinotecan, and oxaliplatin (FOLFIRINOX) regimen has been shown to be superior to the PEFG-regimen.

According to Conroy *et al*[121], FOLFIRINOX is the new standard in the treatment of advanced stage pancreatic cancer. Compared to gemcitabine alone, FOLFIRINOX demonstrated a better objective response rate, progression-free survival, overall survival and one-year survival. While the toxicity levels associated with FOLFIRINOX are greater than those caused by gemcitabine, the effects did not seem to have a significant impact on quality of life. In addition, few toxic deaths have been reported.

Inhibitors of epidermal growth factor receptor (EGFR) have also been tested for treatment of metastatic pancreatic cancer. Cetuximab, an anti-EGFR directed antibody and erlotinib, an oral EGFR tyrosine kinase inhibitor have been tested in several randomized trials. However, Moore *et al*[122] demonstrated that combining gemcitabine with erlotinib is the only targeted-therapeutic agent that has clinical efficacy. Compared with gemcitabine alone, gemcitabine plus erlotinib showed significant decrease in tumor progression and concurrently increased overall survival rates.

Other targeted therapies have focused on targeting

the desmoplastic stroma, one of the key components of pancreatic cancer that may contribute to impaired drug delivery and thus chemotherapy resistance[79]. Nab-paclitaxel is one therapy that has been developed to diminish this stromal tissue network. Studies have demonstrated that albumin interacts with secreted protein acidic and rich in cysteine (SPARC), a matrix glycoprotein that has a role in tumor invasion, facilitating the uptake of paclitaxel by the tumor[123]. Infante *et al*[124] have demonstrated that overexpression of SPARC in peritumoral fibroblasts was a negative prognostic indicator in patients with advanced pancreatic cancer[124]. In a phase I / II trial, Von Hoff *et al*[125] demonstrated the ability of nab-paclitaxel to increase median survival rate in patients with metastatic pancreatic cancer.

Similar to the mechanism of action of nab-paclitaxel, new therapies are being developed that target the peritumoral stroma in order to increase tumor perfusion. One such preclinical strategy inhibits the hedgehog signaling pathway, depleting the stroma and increasing angiogenesis to improve delivery of chemotherapeutic agents to the tumor[126].

Phase II clinical trials have demonstrated a benefit of second-line treatment for patients who are resistant to gemcitabine treatment[127]. Second-line treatments typically consist of fluoropyrimidines in combination with oxaliplatin[128]. Limited data exists on how to treat patients who do not tolerate FOLFIRINOX as a first-line therapy. However, Conroy *et al*[121] have demonstrated the benefit of using gemcitabine-based therapies in these instances.

The primary prognostic indicators for patient survival are both patient and tumor related. Based on the genetic and morphologic heterogeneity that exists within each individual pancreatic tumor, therapy, dose and length of therapy administration will need to be customized for each Individual patient to ensure optimal treatment.

Radiation

Neoadjuvant radiation therapy: Many studies have demonstrated the important roles for chemotherapy and radiation therapy in preventing the recurrence and improving the resectability of pancreatic tumors. While surgery is currently the only potential curative treatment modality for pancreatic cancer, more than 80% of patients who undergo surgical resection will experience tumor recurrence within 12 mo of their procedure[129]. Therefore, a great deal of focus has not only been placed on developing effective neoadjuvant and adjuvant therapies, but also on effective preoperative staging techniques to determine candidates who will benefit most from surgical resection[130]. Since surgery is associated with high rates of morbidity and mortality, many patients do not begin adjuvant therapy until after they have recovered. As a result, there is a long delay before they receive adjuvant therapy. In order to begin more potent treatments earlier and to potentially shrink the tumor before surgery, researchers developed neoadjuvant therapeutic regimens.

Multiple trials of 5-fluorouracil-based chemoradiation have been done to date. At the conclusion of these studies, researchers determined that a combined treatment modality with preoperative rapid-fractionation chemoradiation, Whipple procedure, and intra-operative radiation therapy resulted in minimal toxicity and a small recurrence rate[131]. In a follow-up study, paclitaxel replaced 5-fluorouracil and was used to treat 35 patients who presented with resectable pancreatic tumors[132]. Based on the results of this study, researchers concluded that preoperative paclitaxel-based chemotherapy with rapid-fractionation chemoradiation, Whipple procedure and intraoperative radiation therapy resulted in similar outcomes as the previous study, but toxicity levels were greater than those from 5-fluorouracil. In another study, researchers treated patients who presented with tumors in the head of the pancreas with a neoadjuvant chemoradiation regimen of capecitabine with proton beam radiation[133]. No dose limiting toxicities were observed and the authors concluded that this form of neoadjuvant therapy was feasible. In several other prospective neoadjuvant chemoradiation trials in patients with resectable pancreatic cancers, the rate of resection was high in all studies, ranging from 87%-100%[107,134,135].

Adjuvant radiation therapy: The median survival rate of patients who undergo surgical resection of a pancreatic tumor is 15-22 mo. Only 20% of patients survive for five years following surgery[136]. The most common site for pancreatic cancer recurrence is the retroperitoneum. Therefore, adjuvant therapy is needed to improve patient prognosis. In the United States, adjuvant therapy is currently delivered in the form of chemotherapy, chemoradiotherapy or chemotherapy followed by chemoradiotherapy. Standard adjuvant treatment in Europe is chemotherapy alone. These guidelines were based on previous randomized trials that showed improved survival in patients given adjuvant therapy following surgical resection.

The first prospective trial for adjuvant chemoradiotherapy was conducted by the Gastrointestinal Tumor Study Group in 1985. The trial enrolled patients with resectable pancreatic cancer. The protocol called for external beam radiation delivered with 5-fluorouracil. The patients were then given a maintenance dose of 5-fluoruracil for an additional two years following initial treatment. Patients treated with adjuvant chemoradiation achieved a longer median and 2-year survival rate than those not treated with adjuvant therapy. As a result, adjuvant chemoradiation became the most frequently used adjuvant treatment for resectable pancreatic cancer in the United States.

To further assess adjuvant radiation therapy for resectable pancreatic cancer, the European Study Group for Pancreatic Cancer (ESPAC-1) conducted the largest randomized trial to date in 2004[137]. In order to evaluate the effects of chemoradiotherapy and chemotherapy on patient survival following surgical resection, patients with resectable pancreatic ductal adenocarcinoma were divided into one of four groups: chemotherapy alone;

chemoradiotherapy alone; chemoradiotherapy followed by chemotherapy; or no further treatment. Patients who received chemotherapy followed by chemoradiotherapy had a 5-year survival rate that was 10% less than those who received chemotherapy alone. In addition, patients who received chemotherapy treatment showed a 5-year survival benefit when compared to those who received no chemotherapy. As a result of these findings, the standard of adjuvant treatment in Europe shifted towards chemotherapy only, abandoning postoperative chemoradiation.

A phase Ⅲ trial was conducted by the Radiation Oncology Group and GI Intergroup around the same time as the ESPAC-1 trial[138]. This trial compared the 5-fluorouracil-based chemoradiation to gemcitabine-based chemoradiation. Patients receiving gemicitabine-based chemoradiation had a median survival of 20.6 mo, 3.5 mo more than those given 5-flurouracil-based chemotherapy. The Charite Onkologie Clinical Studies in GI Cancer 001 (CONKO-001) trial in Germany and Austria showed similar median survival in patients given gemicitabine-based chemotherapy alone[139].

Additionally, reports from several institutions, including the Mayo Clinic, Johns Hopkins Medical Center and Virginia Mason University have all reported the benefit of adjuvant chemoradiation therapy following resectable pancreatic cancer compared to those who received surgery alone[140-142].

Management of locally advanced pancreatic cancer: Patients with locally advanced pancreatic cancer achieve little benefit from surgical resection because their cancer meets the criteria for unresectable cancer: (1) distant metastasis and/or pancreatic lymph node involvement; (2) encasement or occlusion of the superior mesenteric vein or superior mesenteric vein/portal vein confluence; and/or (3) direct involvement of the celiac axis, aorta, inferior vena cava, or superior mesenteric artery[143]. As a result, chemoradiation is recommended for these patients based on data from several studies.

A 1981 trial conducted by the Gastrointestinal Tumor Study Group compared the effects of high-dose radiation therapy alone; moderate dose radiation combined with 5-flurorouracil and high dose radiation combined with 5-fluorouracil in 194 patients with locally advanced pancreatic cancer. Researchers found that patients administered 5-fluorouracil in combination with low or high dose radiation showed a greater median survival than those treated with radiation alone[144]. In a follow-up study, the same group demonstrated that chemotherapy, when combined with radiotherapy afforded patients with locally advanced pancreatic cancer a greater median survival when compared to combination chemotherapy alone[145]. These results were verified by the ECOG trial as well, which demonstrated an increased median survival rate in patients treated with gemcitabine and radiotherapy together as opposed to those treated with gemcitabine alone[146].

Although chemoradiation has been shown to provide an increased median survival in patients with locally advanced pancreatic cancer by 9-13 mo, many of these patients progress to the metastatic stage of disease shortly after therapy. Perhaps a better approach to these patients would be to begin a chemotherapy regimen, restage their cancer after completion of initial treatment, and follow up with chemoradiation in patients who do not demonstrate metastatic disease progression. Radiation in these patients could relieve pain associated with disease by slowing local progression.

Stereotactic body radiotherapy: An evolving radiation therapy for treatment of locally advanced pancreatic cancer is stereotactic body radiation therapy. This newer technique uses image guidance to deliver toxic radiation doses directly to tumors. As a result, there is less systemic involvement, and patient outcomes are improved without having to undergo daily treatments. However, the major challenge of this novel therapy is accurately characterizing the tumor in terms of size, number and location. In order to do so, precise diagnostic tests and real-time imaging techniques are used. In addition, each treatment regimen is tailored to each individual patient. To date, scientific literature suggests sterotactic body radiotherapy does slow local progression in patients with locally advanced pancreatic cancer[147-149]. However, it does not increase overall survival rate because patient mortality is due to distant metastases.

Advances in radiation therapy techniques: Over the past decade, major advances in radiation therapy have been in treatment planning and more precise delivery methodologies. One technique, intensity-modulated radiotherapy, decreases systemic toxicities in patients by modifying radiation dose delivery specifically to the tumor sites, sparing surrounding normal tissue[150,151]. Another technique, image-guided readiotherapy, has provided more accurate visualization and real-time tracking of viscerally-located tumors and thus has enabled more precise delivery of high-dose therapeutic beams of radiation to these tumors and prevented adverse effects in normal tissue[152].

In order to improve patient outcome and prolong median survival rate, additional studies are needed to define the optimal role of adjuvant and neoadjuvant treatment in patients with resectable pancreatic cancer. As radiation therapies become more precise and customized to individual patients, it will be necessary to continue to investigate their future role in the treatment of pancreatic adenocarcinoma, especially as a greater understanding of the molecular pathways involved in the carcinogenesis and progression of this disease are understood.

Personalized therapy: In an article published in *Science*, Jones *et al*[77] performed a comprehensive genome assessment on 24 different pancreatic cancers. Results revealed an average of 63 genetic mutations per cancer, spanning 12 separate signal transduction pathways. This study sup-

ports the notion of pancreatic cancer being a genetically heterogeneous malignancy, partially accounting for its notable resistance to therapy as well as varied responses to treatment. Moreover, this finding likely explains why no candidate gene has been identified in the treatment of pancreatic cancer. This heterogeneity will likely dictate an individualized, unique approach for each particular case, which has already shown to be effective against even advanced pancreatic cancer stages. In one such case report, Villarroel *et al*[153] identified Mitomycin C, a DNA-damaging agent, as a highly effective agent by utilizing a xenograft derived from the patient's tumor. Upon administration of this drug, the patient exhibited notable clinical benefits for over three years, despite the tumor previously being gemcitabine resistant. Personalized immune system stimulation may also be a viable option in treatment of unresectable disease. For example, Yanagimoto *et al*[154] incorporated a vaccine containing individualized, reactive peptides with concurrent gemcitabine treatment, noting a significant correlation between immune boosting and survivorship.

Due to the ongoing advances in DNA sequencing, personalized genomic therapy appears more plausible. Moreover, as scientists continue to identify regions of the genome with high potential for tumor-pathogenesis, this method will only become more efficient. Upon identification, cases can be distributed into cohorts based upon their tumor's genetic composition and administered treatment previously demonstrated to be effective in that particular subgroup. This method would not only identify pertinent biomolecules in pancreatic pathogenesis, but also lead to tumor-specific treatment, which is likely necessary if we are to see any significant improvement in the prognosis of pancreatic cancer.

FUTURE PERSPECTIVES

Despite decades of effort by the scientific community to design sophisticated chemotherapeutic and radiation techniques to combat pancreatic ductal adenocarcinoma, less than 5% of patients with this disease have a 5-year survival rate. The majority of patients have a median survival period of 4-6 mo[155,156]. A combination of factors including few early symptoms, few accurate biomarkers for early detection, rapid metastasis to the lymphatic system and distant organs, and few effective treatment options, makes this disease one of the most deadly cancers today[157,158]. Although current therapeutic agents have had limited effects on patient care, there has been substantial advancement in the understanding of the molecular and biological makeup of pancreatic adenocarcinoma. This knowledge has the potential to lead to the development of novel therapies that could significantly improve the lifespan of individuals suffering with this disease.

Such advances in understanding this complex disease have been achieved with genetically-engineered mouse models and patient-derived xenografts. These studies have demonstrated the genetic diversity of pancreatic ductal adenocarcinoma results from successive accumulation of mutations in several primary oncogenes and tumor suppressor genes, leading to its heterogeneity, instability and early tumor metastasis[77]. Pancreatic ductal adenocarcinoma is composed of several compartments. In addition to a mature cancer cell population, some researchers have characterized cancer cells that display stem cell properties and are resistant to chemotherapy and radiation therapy, potentiating their ability to metastasize[56]. Another area of interest is the dense tumor microenvironment that surrounds the pancreatic cancer cells. Composed of collagen I , activated fibroblasts, and inflammatory cells, it has been shown to interact with pancreatic cancer cells in order to foster tumor development, act as a barrier to optimal drug delivery and aid the tumor in invasion and metastasis[59]. Furthermore, this dense stroma creates a hypoxic microenvironment that pancreatic cancer cells thrive in[159]. However, the mechanisms by which these cancer cells adapt to these conditions are currently being identified and may serve as additional therapeutic targets in the near future.

Pancreatic cancer cells

Pancreatic ductal adenocarcinoma most likely originates in the ductal epithelium of pancreatic cells[160]. Neoplastic cells contain one or more of four primary genetic mutations that will ultimately give rise to the invasive form of this disease. Ninety percent of these tumors have mutations in the KRAS2 oncogene, resulting in the activation of proliferative survival signaling pathways. Ninety-five percent have a mutation in the CDKN2A tumor suppressor gene, resulting in the loss of the p16 protein and thus loss of regulation of the G_1-S transition of the cell cycle. An abnormal *TP53* gene has been identified in 50%-75% of characterized cancer cells, allowing cells to avoid DNA damage control checkpoints and subsequently, apoptotic signals. Another 50% have a deleted SMAD4/MADH4 gene, resulting in aberrant signaling by the TGF-β cell surface receptor[153].

One study performed genetic analysis on 24 pancreatic ductal adneocarcinomas and reported that each tumor has an average of 63 clinically relevant genetic abnormalities. While these abnormalities differ from one cancer to another, they all seem to play a role in 12 functional cancer-related pathways[161]. Recently, two studies compared the genetic makeup of distant metastases to their primary metastatic lesions. They found that over time, the distant metastases accumulated additional mutations to those present in the clonal cells from which they arose, adding to the complexity this disease[162]. Such genetic diversity not only results in different prognoses for patients, but also causes individual tumors to respond differently to common therapeutic agents used in treating pancreatic ductal adenocarcinoma[163].

The varying degrees of genetic instability that exist between individual pancreatic ductal adenocarcinomas present a greater need for genomic sequencing of individual tumors, followed by personalized therapies to target spe-

cific genes and pathways that have been altered[164]. Several clinical trials have begun exploring this idea[165]. In order to incorporate this treatment modality into the clinical setting, several criteria must be met: (1) a high quality tumor tissue sample must be attained at the time of diagnosis; (2) sophisticated bioinformatic analysis of the data must be performed to identify the most relevant mutations in each tissue sample; and (3) model systems must be designed to experimentally test varying treatment options to determine the most effective one for the patient. Perhaps the greatest challenge lies in developing a drug once specific genetic abnormalities have been identified.

Pancreatic cancer stem cells

Recently, investigators have characterized pancreatic cancer cells with stem cell properties[162]. Known as pancreatic cancer stem cells, these cells have the ability to regrow new tumors when placed into naïve mouse models and are able to maintain long-term tumorigenic potential[163]. Studies have shown that pancreatic CSCs are not only capable of self renewal, but may also confer therapeutic resistance, and play a role in tumor formation and disease progression[164,165]. In addition, different cancer stem cell populations perform different biological functions. One of the most recent findings has demonstrated that these cells may transition between epithelial and mesenchymal states, contributing to their highly metastitc potential[165]. Therefore, eliminating or inhibiting these CSCs with new therapeutic designs could significantly improve patient outcomes. Current therapies have already been designed to target cancer stem cell-specific antigens in order to inhibit their roles in cell survival, adhesion, self renewal and differentiation. A greater understanding of individual CSC populations and how they interact with one another will enable further progress in the treatment of pancreatic cancer[164,165].

Therapeutic targets of pancreatic ductal adenocarcinoma cancer stem cells include genes located in developmental pathways such as hedgehog, Wnt, Notch, CXCR4 and Met. In addition, targeting apoptotic pathways such as DR5 and nodal-activin could have a significant therapeutic implications. Several preclinical trials have been conducted to target these pathways in models of human pancreatic ductal adenocarcinoma cancer stem cells. By inhibiting these pathways, investigators were able to confer longer-term tumor control when compared to current standard chemotherapeutic regimens, in which tumor regression was significantly shorter-lived. In one recent trial, salinomycin was shown to induce cell death in epithelial-mesenchymal transition-induced cancer stem cells[124].

Due to the heterogeneity of the cancer stem cell population, future drugs designed to target pancreatic ductal adenocarcinoma stem cells may require clinical trials in which therapies are designed specifically for pancreatic tumors in each individual patient. These customized therapies could potentially serve as adjuvant treatment options for patients following pancreatic tumor resection. Similar to previous clinical trial designs, adjuvant therapies targeting cancer stem cells could be given to patients with or without current conventional chemotherapy and/or chemoradiation to determine which option confers the greatest overall survival rate in patients following surgical resection.

Tumor microenvironment

One of the primary characteristics of pancreatic ductal adenocarcinoma is the dense stroma surrounding the pancreatic cancer cells. Composed of fibroblasts, collagen I and other fibrillar elements, this desmoplastic reaction has become a primary target of current drug therapies[125]. The key players in the formation and turnover of this dense stroma are pancreatic stellate cells. Certain growth factors [TGF-β1, platelet-derived growth factor (PDGF) and fibroblast growth factor] activate these cells to myofibroblasts. Not only do these activated myofibroblasts secrete components of the extracellular matrix, but they are also responsible for the poor vascularization of the pancreatic tumor[166,167]. In addition to forming a mechanical barrier around the pancreatic cancer cells, the stroma has an important role in tumor formation, progression, invasion and metastasis[168]. Many proteins expressed by stromal cells have been directly correlated with poor prognosis and resistance to current therapies [Cox-2, PDGF receptor, VEGF, stromal-derived factor, chemokines, integrins, secreted protein acidic and rich in cysteine (SPARC), and hedgehog elements].

Pre-clinical models have demonstrated that targeting these receptors and enzymes is associated with antitumor effects. Perhaps one of the most promising targets to date is the hedgehog signaling pathway. Some studies have demonstrated that targeting smoothened resulted in a depletion of the stroma and thereby increased delivery of gemcitabine to the tumor cells[169]. Another target for therapeutic trials has been SPARC (osteonectin) and hyaluronic acid. SPARC is an extracellular matrix protein that plays a role in collagen turnover in the dense stroma. It is associated with invasion and metastasis in pancreatic ductal adenocarcinoma, and thus poor prognosis in patients with elevated levels[170]. As mentioned previously, SPARC is the target of the albumin-bound chemotherapy agent, nab-paclitaxel. Phase Ⅰ/Ⅱ clinical trials have shown that administration of this drug breaks down the stroma and improves delivery of the chemotherapeutic agent to the site of the tumor[171]. In addition, in a mouse model of pancreatic ductal adenocarcinoma, investigators demonstrated that administration of pegylated hyaluronidase eliminated hyaluronic acid content, thereby relieving pressure on the blood vessels surrounding the tumor and allowed for increased perfusion of the chemotherapeutic agent to the site of the tumor[172].

The immunosuppressive nature of the tumor microenvironment has been another stromal characteristic targeted by recent therapeutic development. Using a CD40 antibody combined with gemcitabine chemotherapy, researchers have attempted to reverse immune suppression and drive antitumor T-cell responses in patients with non-resectable pancreatic ductal adenocarcinoma. Studies

have shown that this agent results in tumor regression by stimulating tumor macrophages to attack and deplete the pancreatic cancer stroma[173].

To date, targeting pancreatic ductal adenocarcinoma has proved most effective when treating patients with locally advanced disease, especially patients with tumors characterized by wild-type DPC4. These tumors are known to be less prone to metastasis and possess higher stromal content. Other tumors, especially those in late stages of the disease, characterized by distant metastases, have not been effectively treated with current stromal-targeting therapeutic agents. This is due to the fact that although pancreatic ductal adenocarcinoma has a rich and hypovascularized stroma, metastases arising from this cancer do not and are not different from other tumors. Therefore, patients who may benefit most from treatment with agents targeting the dense stroma microenvironment would be those with resectable tumors that have not progressed to the advanced stages of disease[174].

Metabolic pathways

Another conventional way to target pancreatic ductal adenocarcinoma would be to inhibit its major metabolic pathways. In order to do so, researchers would need to prevent its supply of glucose and glutamine; interrupt the pathways that enable it to exist in a hypoxic environment[175]; and prevent its ability to digest intracellular organelles for energy[176].

Investigators have identified several key metabolic enzymes to target [hexokinase, pyruvate kinase, lactate dehydrogenase A (LDHA) and ampicillin-activated protein kinase (AMPK)]. Several preclinical trials have demonstrated the anti-tumor effects of agents directed against these enzymes. One study demonstrated a potential clinical application for the LDHA inhibitor, FX11. By blocking the conversion of lactate to pyruvate in cells with p53 mutations, FX11 has antitumor potential. However, to date, there are only two therapies that have shown potential for targeting pancreatic ductal adenocarcinoma metabolism. One of these medications, metformin, is an activator of AMPK. It has been shown to decrease the potential for patients with diabetes to develop pancreatic cancer and to increase survival in diabetic patients with this disease[177,178]. The other drug used to target the metabolic pathways of is rapamycin. An inhibitor of mTOR, rapamycin has been shown to decrease glucose uptake by reducing levels of Glut1 in pancreatic cancer[179,180].

In order to inhibit autophagy, a significant mechanism for pancreatic cancer cell survival, investigators have used chloroquine, the antimalarial drug[181]. In preclinical trials with both allografts and xenografts, chloroquine has been shown to decrease tumorigenesis in a transgenic model and is currently being tested in clinical trials.

CONCLUSION

A greater understanding of the molecular and cellular makeup of pancreatic cancer over the past four decades has resulted in innovative therapeutic designs to target this aggressive malignancy. We now know that pancreatic cancer is a dynamic, heterogenous and genetically unstable tumor that results from successive mutations early in disease and gives rise to metastases that continue to garner mutations as they travel to distant locations. An equally important understanding of the role the peritumor microenvironment, composed of a dense desmoplastic stroma, plays in tumor development, metastases and as a barrier to chemotherapy delivery has been elucidated over the years. More recently, a role for pancreatic cancer stem cells in resistance to chemotherapy and radiation therapy was discovered. In addition, a deeper understanding of the metabolic pathways responsible for adaptation of pancreatic cancer cells to hypoxic environments has significant implications for future therapeutic development.

While some of these discoveries have resulted in novel therapeutic targets and treatment strategies and others are currently being tested in preclinical trials, efficient and effective drug development to combat pancreatic ductal adenocarcinoma is a necessity for the future. The majority of clinical trials have been conducted in patients with advanced stage disease. In the future, it will be necessary to design clinical trials to enroll patients with earlier stages of pancreatic cancer in an attempt to cure their cancer before it can metastasize.

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P- Reviewer: Liu X, Muscarella P, Kurniali PC **S- Editor**: Ma YJ
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World Journal of Gastroenterology

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DOI: 10.3748/wjg.v20.i32.11160

World J Gastroenterol 2014 August 28; 20(32): 11160-11181
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (14): Pancreatic cancer

Complex role for the immune system in initiation and progression of pancreatic cancer

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Author contributions: Inman KS performed the research; Inman KS and Francis AA analyzed the data; Inman KS and Murray NR wrote the review.

Supported by The National Institute of Health (NIH/NCI R01CA140290-3 to Murray NR)

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Received: October 29, 2013 Revised: February 27, 2014
Accepted: April 15, 2014
Published online: August 28, 2014

Abstract

The immune system plays a complex role in the development and progression of pancreatic cancer. Inflammation can promote the formation of premalignant lesions and accelerate pancreatic cancer development. Conversely, pancreatic cancer is characterized by an immunosuppressive environment, which is thought to promote tumor progression and invasion. Here we review the current literature describing the role of the immune response in the progressive development of pancreatic cancer, with a focus on the mechanisms that drive recruitment and activation of immune cells at the tumor site, and our current understanding of the function of the immune cell types at the tumor. Recent clinical and preclinical data are reviewed, detailing the involvement of the immune response in pancreatitis and pancreatic cancer, including the role of specific cytokines and implications for disease outcome. Acute pancreatitis is characterized by a predominantly innate immune response, while chronic pancreatitis elicits an immune response that involves both innate and adaptive immune cells, and often results in profound systemic immune-suppression. Pancreatic adenocarcinoma is characterized by marked immune dysfunction driven by immunosuppressive cell types, tumor-promoting immune cells, and defective or absent inflammatory cells. Recent studies reveal that immune cells interact with cancer stem cells and tumor stromal cells, and these interactions have an impact on development and progression of pancreatic ductal adenocarcinoma (PDAC). Finally, current PDAC therapies are reviewed and the potential for harnessing the actions of the immune response to assist in targeting pancreatic cancer using immunotherapy is discussed.

Key words: Immune system; Pancreatitis; Pancreatic ductal adenocarcinoma; Immunosuppression; Immunotherapy; Inflammation

Core tip: The development and progression of pancreatic cancer is heavily influenced by the immune response. Inflammation of the pancreas (pancreatitis) is a significant risk factor for pancreatic cancer. Immune cells recruited to the inflamed pancreas release additional cytokines and potentiate damage to the tissue. Pancreatic cancer is characterized by profound immune suppression thought to be caused by signals originating from the tumor cells. Additionally, a subset of immune cells has been shown to support the growth of pancreatic cancer cells. Novel therapies for pancreatic cancer aim to utilize this unique immune environment to target this deadly disease.

Inman KS, Francis AA, Murray NR. Complex role for the immune system in initiation and progression of pancreatic cancer. *World J Gastroenterol* 2014; 20(32): 11160-11181 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11160.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11160

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer, and with a 5-year survival rate of 6%, it is one of the deadliest cancers worldwide[1]. The development and progression of PDAC is strongly influenced by the presence of inflammation[2]. Inflammation of the pancreas (pancreatitis) is a strong risk factor for PDAC development[3,4], and has been described as a critical factor in the initiation[5] and maintenance[6,7] of pancreatic disease. Additionally, PDAC is characterized by marked immunosuppression[6], which is thought to promote tumor progression and invasion.

This review highlights the role(s) of the immune response in the development of PDAC, focusing primarily on inflammation. Inflammatory conditions of the pancreas that can lead to increased risk for PDAC (pancreatitis), as well as the role of the immune response in the progressive stages of pancreas disease development, are discussed. Data from clinical studies and rodent models are integrated to present an up-to-date consensus of the role of inflammation in the initiation and progression of pancreatic cancer.

IMMUNE SYSTEM: A BRIEF OVERVIEW

The immune system is characteristically activated in response to infection by a foreign pathogen. In the case of cancer, tumor-specific antigens are recognized by the immune system, turning the cancer cell, in essence, into a foreign pathogen[8]. This allows the immune system to act as an extrinsic tumor-suppressor system. However, over time the chronic immune response to the tumor drives immunoselection of tumor cells that are able to thrive in an immunocompetent environment[9], much like the resistant bacterial strains that emerge as a result of exposure to antibiotics.

The mechanism by which the immune system can initially protect a host from tumor growth, but in some cases subsequently promotes cancer progression, is termed cancer immunoediting[10]. Cancer immunoediting is a dynamic process consisting of three phases, (1) elimination, when the immune system overcomes and eliminates the tumor before it can progress to a clinically relevant disease; (2) equilibrium, when the immune system does not eliminate the tumor, but controls tumor growth; and (3) escape, which occurs when the tumor has overcome the immune system and progresses to a clinically apparent disease. This third stage is generally seen as a failure in the adaptive immune system to provide long-term protection from tumor development due to selection of less immunogenic tumor cell variants during the equilibrium stage. Additionally, tumor escape can be facilitated by active immunosuppression induced by the tumor itself or some form of immune compromise or immune deficiency[11]. It is through this process that the cells of the immune system can act as both friend and foe to the body in the face of cancer.

Innate immunity

The innate immune system is composed of those immune cells that are already present in the body and can be immediately recruited to a site of infection during the process of "inflammation". Innate immune cells include granulocytes, macrophages, mast cells, natural killer cells (NKCs) and dendritic cells (DCs). Table 1 reviews the mechanism(s) of action attributed to the innate immune cells in both the normal immune response and during the process of cancer development. Neutrophils are by far the most abundant granulocytes in the body and typically one of the first cell types to respond; they secrete cytokines and chemokines, modulating other cells in the immune response[12]. Macrophages, another major cell type of the innate immune response, remove dead or dying cells and associated debris *via* phagocytosis, as well as play a role in the adaptive immune response. Macrophage maturation in response to various signals produces one of two cell types, M1-polarized cells, which initiate the inflammatory response, and M2-polarized cells, which restrain the inflammatory response[13]. M2-polarized macrophages are immunosuppressive and can limit adaptive immunity by inhibiting T-lymphocyte proliferation, thus impeding the T-lymphocyte response[14].

In the context of cancer, two types of macrophages emerge, tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSC). Both cell types are similar to M2 macrophages and are recruited by tumor cells to thwart the anti-tumor immune activity. TAMs inhibit T-lymphocyte responses[15] and secrete cytokines that promote the tumor phenotype and metastasis[16,17]. In addition to their ability to directly induce T-lymphocyte apoptosis[18], TAMs produce arginase-1[19], a metalloenzyme that metabolizes and depletes the environment of arginine, an essential compound for T-lymphocyte proliferation[20,21].

The second type of immunosuppressive macrophage often found in the tumor microenvironment is the MDSC. Similar to TAMs in function, they differ mainly by their cell surface markers. MDSCs have been shown to exhibit powerful immunosuppressive properties, in part through production of reactive oxygen species and peroxynitrite[22,23], expression of arginase-1[24,25], induction of regulatory T-lymphocytes (Tregs)[26], and depletion of available cysteine, another amino acid required by T-lymphocytes[27].

NKCs are circulating immune cells that are able to kill target cells *via* induction of programmed cell death. NKCs have been shown to eliminate tumor cells, and treated cancer patients with high circulating NKCs have significantly longer metastasis-free survival[28]. The gap between the innate and adaptive immune systems is bridged by DCs[29]. Activated, antigen-presenting-DCs travel to the lymphoid organs where they interact with, and activate, B- and T-lymphocytes. In this way, activation of DCs by foreign pathogens can lead to the activation of both the innate and adaptive immune responses, allowing the body to fully respond to the perceived threat[30].

Table 1 Major functions of the innate immune cells in both inflammation and cancer

Inflammatory cell	Immune function	Role in cancer
Neutrophil	Secretion of cytokines/chemokines to modulate other cells in the immune response	Maintenance of pro-angiogenic phenotype[242]
	Release of cytotoxic granules	Suppression of anti-tumor immunity[243]
	Phagocytosis	Promotion of metastasis[244]
Mast cell	Release of cytotoxic granules	Suppression of anti-tumor immunity[245]
	Enhancement of immune cell recruitment	Stimulation of angiogenesis[152]
	Permeabilization of blood vessels	Direct stimulation of cancer cell growth[246]
		Secretion of mitogenic factors[157]
Macrophage	Phagocytosis	
	Promotion of T-lymphocyte activation	
M1	Initiation of immune cell response	
	Antigen presentation	
M2	Wound repair	
	Immunosuppression	
	Tissue remodeling	
	Resolution of immune response	
TAM		Secretion of Arginase-1[19]
		Support of Treg activation[247,248]
		Promotion of angiogenesis[16]
		Enhancement of tumor metastasis[17]
MDSC	Suppression of NKC and T-lymphocyte activation	Production of ROS[22]
		Secretion of peroxynitrite[23]
		Secretion of Arginase-1[24,25]
		Induction of Treg[26]
		Depletion of cysteine[27]
NKC	Release of cytotoxic granules	Tumor cytotoxicity[28]
DC	Antigen presentation	

TAM: Tumor-associated macrophage; MDSC: Myeloid-derived suppressor cell; NKC: Natural killer cell; DC: Dendritic cells.

Adaptive immunity

Innate immunity is a powerful first defense against invading pathogens; however, it is most effective against cells bearing antigens that are common to many pathogens, and can be subverted by quickly evolving cell types, as in the case with cancer cells. The adaptive arm of the immune system is responsible for controlling those pathogens that have overcome the innate response and can provide long-lasting immunity against specific infectious agents.

T- and B-lymphocytes comprise the adaptive arm of the immune response. T-lymphocytes are grouped into classes based on their cell-surface proteins which mediate distinct effector functions. Cytotoxic T-lymphocytes express CD8 and are responsible for killing cells expressing foreign antigen by activating the target cell's apoptosis program, leading to subsequent cell death[30]. Helper T-lymphocytes express CD4 on their surface and assist in the activation of $CD8^+$ T-lymphocytes, B-lymphocytes and macrophages *via* secretion of specific cytokines, thus extending their function across both the innate and adaptive immune responses[30]. A subset of $CD4^+$ lymphocytes, Tregs, protects the body from autoimmune responses. Tregs suppress T-lymphocyte activation in a cytokine independent, cell-contact-dependent manner[31].

B-lymphocytes are one of the main cell types responsible for the body's ability to mount a long-term pathogen-specific response. Each B-lymphocyte produces a single species of antibody, and once activated, proliferates into an antibody-secreting effector cell. It is largely through the action of B-lymphocytes that the body is able to maintain an immunological memory and initiate and immediate response to foreign pathogens it has already encountered[32].

Although inflammation serves to protect the body from harm, it also plays a major role the development of disease, including cancer[33]. Pancreatic cancer, in particular, is heavily influenced by the inflammatory response associated with pancreatic injury and disease. Pancreatitis, or inflammation of the pancreas, is a relatively common condition that often leads to irreversible pancreatic damage and leaves the pancreas vulnerable to the development of neoplastic disease.

PANCREATITIS

The pancreas is comprised of both exocrine (acinar and ductal cells) and endocrine (islets of Langerhans) cells. The exocrine pancreas functions to produce and secrete digestive enzymes into the small intestine whereas the endocrine pancreas is primarily responsible for producing hormones crucial for glucose homeostasis. Dysfunction of the pancreas due to disease or injury can lead to impaired digestion, hypoglycemia and diabetes[34]. Acute pancreatitis (AP) is one of the most commonly diagnosed gastrointestinal diseases, with over 200000 patients admitted to the hospital each year[35]. Patients typically present with acute abdominal pain which may be accompanied by nausea and vomiting, and display increased serum concentrations of amylase and lipase[36]. Pathologically, AP

presents as acinar degranulation, increased occurrences of autophagosomes, formation of dilated acinar lumina and autodigestive fat necrosis[37]. Risk factors for AP include alcohol consumption, gallstones, and smoking, however many cases are idiopathic[38-41]. Although the clinical symptoms of AP often resolve completely, more severe cases can lead to serious complications and even death in a small percentage of patients[42]. Complications associated with AP include pancreatic necrosis[43], infection leading to sepsis[44,45], and systemic inflammatory response syndrome (SIRS) leading to distant organ damage and failure (multiple organ dysfunction syndrome, MODS)[46]. These complications significantly increase the risk of mortality from AP. Additionally, recurrent bouts of AP lead to fibrosis/damage and chronic pancreatitis (CP), a risk factor for the development of pancreatic cancer[4,47].

CP is characterized by acinar loss, extensive fibrosis and immune cell infiltrate, and is a strong risk factor for pancreatic cancer[47-49], which may develop 10-20 years following CP diagnosis[3]. Although CP shares many of the same risk factors, causes, and symptoms as AP, the clinical presentation differs dramatically. Serum levels of pancreatic enzymes amylase and lipase are elevated in AP due to the acute damage caused to the pancreas. In contrast, these enzymes are either normal or only mildly elevated in CP[50]. The chronic inflammation that is the hallmark of CP leads to permanent damage and loss of pancreatic function, leading to diabetes and pancreatic insufficiency[51]. Comprehensive reviews of the diagnosis and etiology of these diseases, as well as factors that distinguish CP from AP, are available[52-54].

Studies from both clinical cases and rodent models of pancreatitis have contributed to the understanding of the inflammatory response to AP and associated syndromes. AP is thought to originate with uncontrolled activation of pancreatic acinar cells and release of digestive enzyme stores leading to autodigestion of the pancreatic cells. This autodigestion releases cellular contents, triggering the recruitment of inflammatory cells. Those inflammatory cells release cytokines and other modulating factors that can amplify the inflammatory response, causing systemic inflammation that can progress to SIRS and MODS. The events responsible for initiating the premature activation of digestive enzymes are not fully understood, but include trypsin auto-digestion[55], generation of reactive oxygen species, disturbances in microcirculation[56], calcium overload, and leukocyte overstimulation[57], and are reviewed in detail elsewhere[58-61].

There is a close relationship between the inflammatory response to AP and clinical severity of the disease[62]. Indeed, overstimulation of leukocytes, specifically neutrophils, results in systemic activation and is thought to be a major cause for severe AP-associated death[57,63]. Many animal models for AP exist in which pancreatitis is induced by pancreatic injury or surgical blockage. While insights into the possible mechanisms of the immune response in the pancreatic disease process can be gained from these models, they do not exactly replicate the clinical disease. Additionally, it should be noted that whereas rodents are the model of choice to study the immune system and its interaction with organ systems, significant differences exist between the human and rodent immune systems in the balance of leukocyte subsets, and in the expression of inflammatory mediators, cytokines and cytokine receptors, as well as the significantly different immunological environments occupied by either species[64]. For these reasons, animal models of pancreatitis will be addressed separately in this section of the review.

Inflammatory response to AP: Clinical findings

AP is characterized by early recruitment and activation of polymorphonuclear cells, the majority of which are neutrophils[57]. Activation of neutrophils can be identified by rising levels of serum neutrophil elastase in the early course of AP, typically within the first two days of diagnosis[62,65,66]. This is accompanied by the detection of metabolically hyperactive granulocytes in the pancreatic tissue of AP patients within 48 h of admission to the hospital, suggesting that granulocyte activation is an early AP event[67]. Neutrophil recruitment is followed by recruitment of the macrophage-monocyte system in the subsequent 2-3 d, as determined by rising levels of C-reactive protein (CRP) in the serum[62,68]. As elastase and CRP are released by neutrophils and macrophages, respectively, the presence of these proteins in the blood of pancreatitis patients is used as an indirect indicator of the recruitment and activation of these inflammatory cells in the pancreas.

Complications associated with AP can be grouped into two phases: immune overactivation and immune suppression. In the first phase, control is lost over the local inflammatory response, leading to excessive and uncontrolled systemic activation of inflammatory cells and mediators[69]. This often leads to the development of SIRS and MODS, and is associated with death within one week of disease[70]. In a subset of patients, the body responds to systemic inflammation with compensatory anti-inflammatory response syndrome (CARS)[71]. CARS initiates the second phase of complications associated with AP and can lead to immune deficiency or suppression, rendering the body susceptible to infection. These patients go on to develop AP-associated infections[44] that are associated with excessive CARS.

Systemically, a decrease in circulating lymphocytes, including B- and T-lymphocytes (both $CD4^+$ and $CD8^+$), as well as NKCs, is often seen in AP[67,72,73]. A decrease in circulating lymphocytes is associated with more severe disease and is often predictive of AP-associated systemic infection[74-76]. Kylänpää-Bäck *et al*[77] demonstrated a significant decrease in monocyte surface expression of human leukocyte antigen-DR (HLA-DR), a hallmark for systemic immunosuppression, in the first 24-48 h following AP diagnosis. Many independent studies have confirmed that a decrease in serum lymphocytes, as well as monocyte HLA-DR, correlates with more severe disease and increased mortality[73-75,77,78].

It is important to understand that the clinical timeline of inflammatory response to AP is a relative concept, as

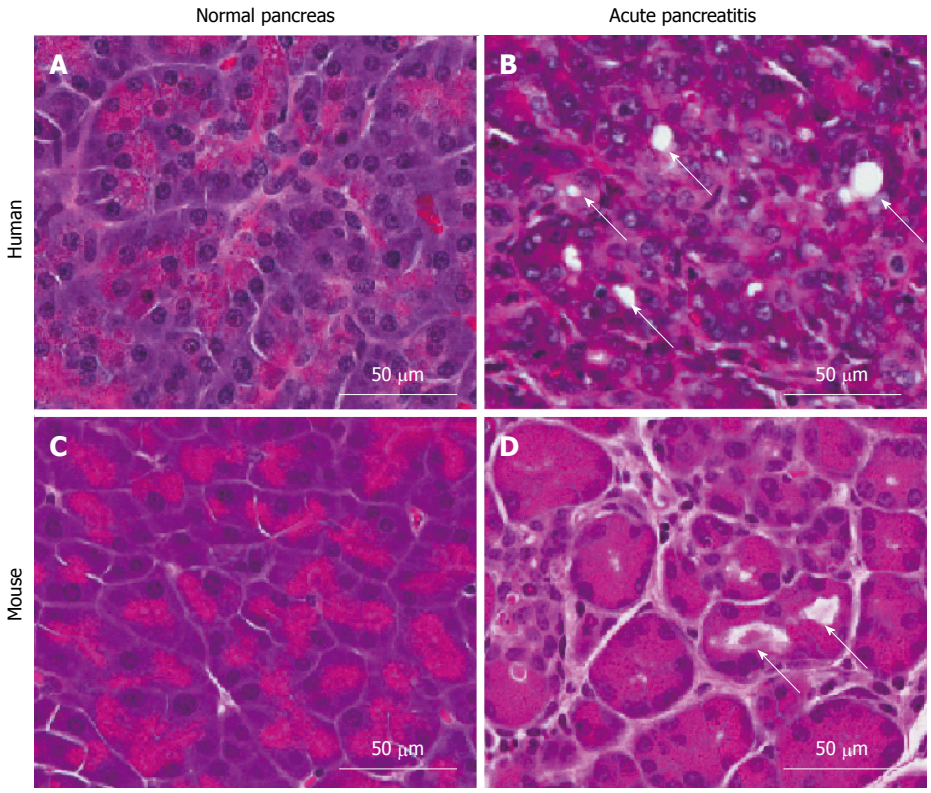

Figure 1 Comparison of histology of human and mouse pancreatic tissue. A: Normal human pancreas; B: Human acute pancreatitis (AP); C: Normal mouse pancreas; D: Mouse AP. Arrows indicate acini with dilated lumina, as commonly seen in AP.

data is dependent on the timing of the patient's admission to the hospital. Additionally, as biopsies are not routinely performed on AP patients, a detailed histological analysis of the pancreatic tissue is often not available. It is these limitations of clinical analysis that led to the use of rodent models in an attempt to more fully understand the mechanism of disease initiation and progression.

Animal models of AP

Several animal models have been developed to study the complex interactions that occur between the pancreatic epithelium and the many inflammatory cell types recruited to the pancreas. These models can be used to more precisely map the time course of the inflammatory response in AP, as well as to determine the mechanism(s) of damage and recovery in order to identify potential therapeutic targets. Although many species have been used to evaluate AP, this review will focus on data generated using rodents. Rodent models are used most often because of their cost-effectiveness and ease of characterization and genetic manipulation; however, no one model completely recapitulates all components of human disease.

The most common method of modeling pancreatitis in rodents is secretagogue hyperstimulation leading to premature intrapancreatic activation of digestive proteases. In this model, administration of high concentrations of the intestinal hormone cholecystokinin, or its molecular ortholog, caerulein, leads to autodigestion of the pancreas[79] and pancreatitis-like pathology including vacuolization, edema, acinar degranulation, dilated acinar lumina, necrosis, lung injury, and cytoplasmic destruction of pancreatic acini[80,81]. Figure 1 shows the histological similarities between human AP and caerulein-induced rodent AP. Another model for AP is surgical ligation of the pancreatic duct. Although this method was designed to mimic clinical gallstone-induced pancreatitis, it often produces a milder form of the disease and is more technically demanding and invasive[82]. Other models of AP include administration of high concentration of L-arginine leading to acinar necrosis[83], feeding of a choline-deficient diet, leading to severe necrotizing pancreatitis[84,85], and overstimulation of the immune system using bacteria or toxins[86].

Inflammatory response to AP: Animal models

Animal models of AP allow histological analysis of all stages of the disease, providing much information regarding the pathogenesis of AP. Although induced AP can present differently depending on animal model utilized, nearly all models result in a recruitment of neutrophils within hours of treatment (Figure 2), thus confirming neutrophils as one of the first responders to pancreatic damage[81,87-91]. Neutrophils have been shown to mediate systemic remote organ injury and death in a murine model of hemorrhagic pancreatitis[92]. Significant macrophage infiltration to the pancreas is observed shortly after caerulein-induced AP (Figure 2), and macrophage-derived macrophage-inflammatory protein-2 (MIP-2) is known to play a role in progression of AP through attraction of leukocytes, promoting tissue injury[93]. Therefore, results

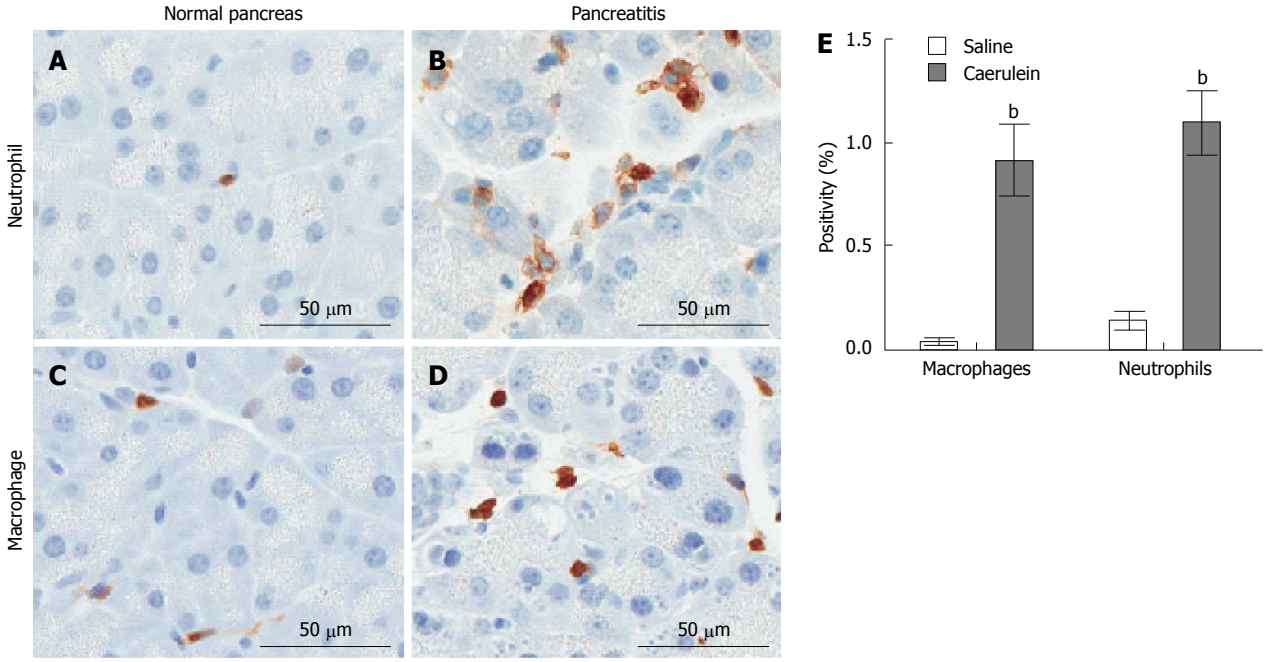

Figure 2 Induction of innate immune response in a mouse model of acute pancreatitis. Mice were injected intraperitoneally with 50 μg/kg caerulein or saline hourly for 7 h (Mayo Clinic IACUC protocol A48510). The pancreas was isolated one hour following the last injection and analyzed immunohistochemically for the presence of neutrophils (Ly6B.2: AbSerotec) or macrophages (Mac-2; Cedarlane Diagnostics). A: Neutrophil infiltration in saline-treated mice; B: Neutrophil infiltration in caerulein-treated mice; C: Macrophage infiltration in saline-treated mice; D: Macrophage infiltration in cearulein-treated mice; E: Stained slides were imaged using ScanScope XT (Aperio, Vista, California) and immunohistochemical staining was quantified using the Aperio ImageScope reader. Mean ± SD is plotted for each group of $n \geq 4$ mice, $^{b}P < 0.01$ *vs* saline group.

from caerulein-treated mice are consistent with clinical data that macrophages also contribute to the early inflammatory response to AP[93].

Mast cells are present in the normal pancreas and undergo degranulation upon induction of AP[87,94]. Inhibition of mast cell degranulation decreases pancreatic inflammation without affecting pancreatic damage[94], suggesting that mast cells primarily play a role in releasing or activating additional inflammatory mediators. DCs are rare in the normal pancreas, but pancreata of caerulein-treated mice exhibit a significant increase in mature DCs. These DCs are crucial for pancreatic viability during injury, as their depletion during caerulein- and L-arginine-induced AP leads to massive pancreatic cell death[95].

A distinct decrease in B- and T-lymphocytes is seen in serum from patients with AP, suggesting a systemic inhibition of the immune system. In support of impaired cell-mediated immunity, interleukin (IL)-2, a product of T-lymphocytes, is decreased in mononuclear splenic cells in a murine model of AP[96]. However, it has been theorized that decreases in circulating lymphocytes are not due to immune suppression but instead to a redistribution of lymphocytes from the blood pool to the pancreas[67]. This concept is difficult to test clinically, as most AP patients will not undergo pancreatic biopsy. In support of this theory, Demols *et al*[97] demonstrated that T-lymphocytes, specifically $CD4^+$ cells, are increased in the murine pancreas following induced AP. This study showed that T-lymphocytes have a role in mediating tissue injury, as depletion of $CD4^+$ T-lymphocytes, or elimination of T-lymphocytes using genetic models, reduced the severity of AP, and this injury was reversible by a T-lymphocyte transfer[97].

Role of cytokines in AP

AP is characterized by excessive recruitment and activation of leukocytes within the pancreas, and in many cases, other organs. Recently, a theory has emerged that the damaged pancreatic epithelium itself is responsible for expression of the first wave of inflammatory mediators, effectively launching the inflammatory cascade that leads to recruitment and activation of immune cells. This is supported by the fact that acinar cells are capable of producing a number of inflammatory mediators in response to damage or noxious stimuli[98-100]. When activated or stressed, isolated acinar cells have been shown to express cytokines[101-104] and chemokines[105] (Table 2 and Figure 3). Detailed reviews regarding the major roles of these mediators in AP and associated conditions are available[106-108]. In addition, activated immune cells secrete numerous chemotactic factors which are capable of perpetuating the immune cell activation cascade.

IL-1β and tumor necrosis factor (TNF) α production by pancreatic cells is thought to be a relatively early event in the activation of the cytokine cascade in AP, with IL-6 secretion occurring later in the disease process[109]. Serum IL-6 correlates with disease severity in humans[110,111] and is useful as an early diagnostic marker of AP[112,113]. In a rat model of pancreatitis, TNFα levels were shown to rise over time following induction of pancreatitis[114], and neutralization of TNFα *via* antibody improved all aspects of pancreatitis (elevated serum amylase, hematocrit, ascites) supporting an important role for TNFα in the

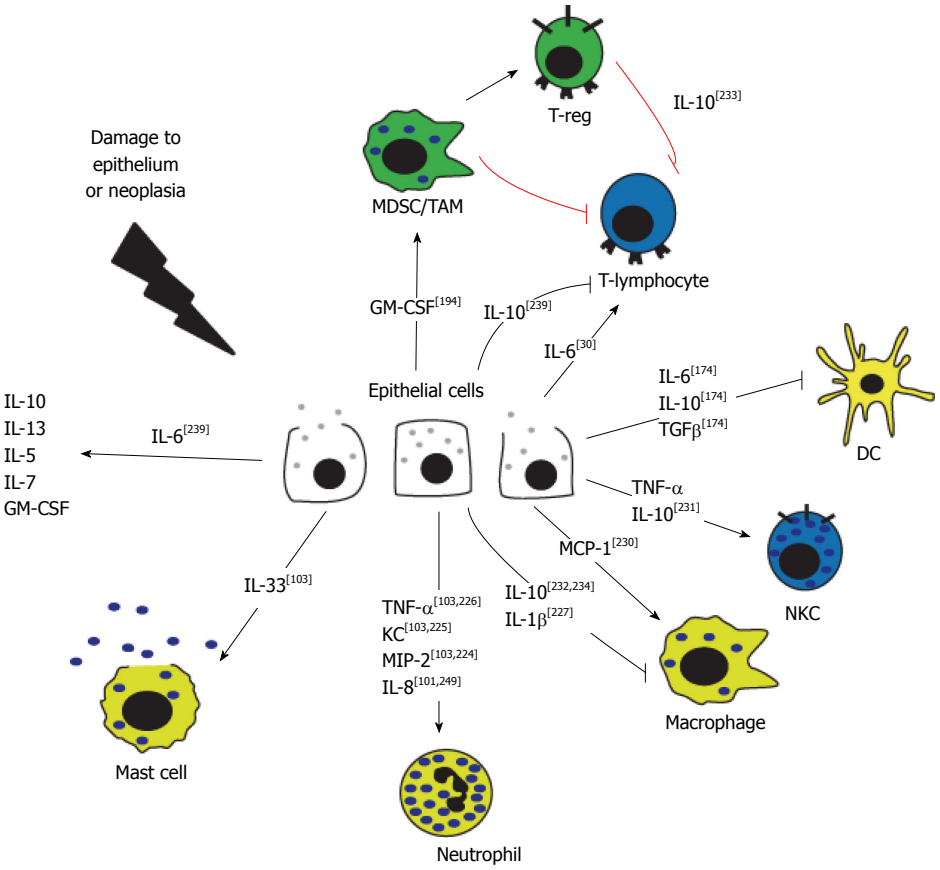

Figure 3 Cytokine signaling in the pancreatic epithelium. In response to damage or disease, the pancreatic epithelial cells release cytokines and chemokines. Activated immune cells secrete numerous chemotactic factors in response to, and in addition to, those secreted by the epithelial cells. TNF: Tumor necrosis factor; IL: Interleukin; MDSC: Myeloid-derived suppressor cells; TAM: Tumor-associated macrophage.

Table 2 Cytokines released during pancreatic injury

Cytokine	Expressed by	Acts on
GM-CSF	Acini[194]	Neutrophils[231], MDSCs[194]
IL-1β	Acini[104,238], activated macrophages[237], neutrophils[236]	Macrohpage (inhibition)[227]
IL-6	Acini[239], monocytes[30], macrophages[30], endothelial cells[30] fibroblasts[30], smooth muscle cells[30], IL-1b[30], TNFα[30]	CRP[228,229], T-lymphocyte[30], DCs (inhibition)[174]
IL-8	Acini[105], IL-1[241], TNFα[241], macrophages[241], neutrophils[235]	Neutrophils[223]
IL-10	Acini[240], Treg[233]	T-lymphocytes (inhibition)[233], DCs (inhibition)[174], macrophages (inhibition)[232,234]
IL-12	Activated macrophages[231], dendritic cells[30]	NKCs[231]
IL-33	Acini[105]	Mast cells[103]
KC	Acini[105], monocytes and neutrophils[250]	Neutrophils[225]
MCP-1	Acini[105]	Macrophages[230]
MIP-2	Acini[105], activated macrophages and neutrophils[93]	Neutrophils[224]
TNFα	Acini[105], activated macrophages[99], neutrophils[236], mast cells[226]	Neutrophils[226], NKC[231]

IL: Interleukin; MCP-1: Monocyte chemotactic protein-1; MIP-2: Macrophage inflammatory protein 2; TNFα: Tumor necrosis factor-α; MDSC: Myeloid-derived suppressor cell; NKC: Natural killer cell; DC: Dendritic cells.

pathogenesis of AP[114]. Blockade of IL-1 signaling using IL-1 receptor antagonist (IL-1ra) attenuates the rise in both IL-6 and TNFα, as well as lessens pancreatic damage in the context of AP, supporting early expression of IL-1 and confirming its role as an important mediator for subsequent cytokines[99].

IL-8 is another cytokine implicated in the early stages of the disease process. IL-8 is an established secretory product of activated macrophages[115], but has also been detected in the pancreatic epithelial cells of clinical and pre-clinical pancreatitis tissue[101,102], suggesting a damaged pancreatic epithelium as a possible source of IL-8. Gross *et al*[116] determined that IL-8 in the serum of pancreatitis patients correlates with disease severity, and showed a significant positive correlation between serum IL-8 and neutrophil elastase, a marker of neutrophils found upregulated in the early stages of AP. As IL-8 is a potent neutrophil activator[117], infiltration of neutrophils may be initiated by

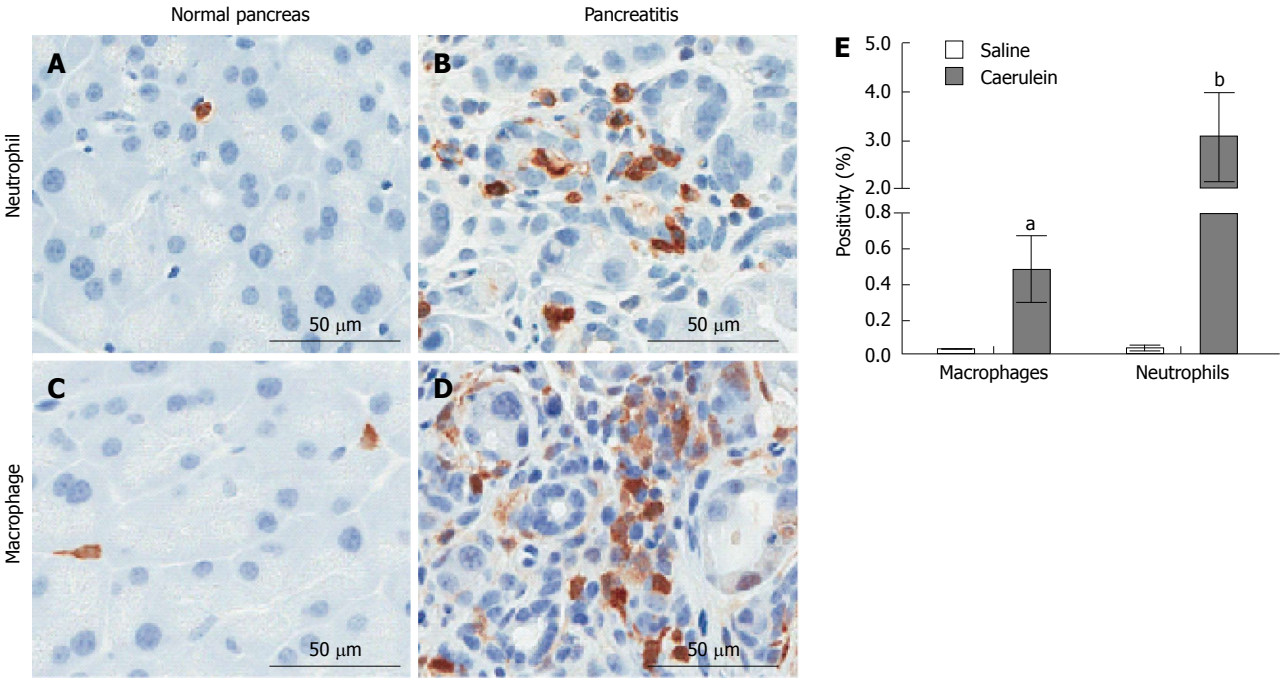

Figure 4 Induction of innate immune response in a mouse model of chronic pancreatitis. Mice were injected intraperatoneally with 250 μg/kg caerulein or saline twice daily, six days per week for two weeks (Mayo Clinic IACUC protocol A48510). The pancreas was isolated 24 h following the last injection and analyzed immunohistochemically for the presence of neutrophils (Ly6B.2: AbSerotec) or macrophages (Mac-2; Cedarlane Diagnostics). A: Neutrophil infiltration in saline-treated mice; B: Neutrophil infiltration in caerulein-treated mice; C: Macrophage infiltration in saline-treated mice; D: Macrophage infiltration in cearulein-treated mice; E: Stained slides were imaged using ScanScope XT (Aperio, Vista, California) and staining was quantified using the Aperio ImageScope reader. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* corresponding saline treated control. Mean ± SD is plotted for each group of $n \geqslant 5$ mice.

secretion of IL-8 from the pancreatic parenchyma. Resident and infiltrating macrophages can also secrete IL-8, recruiting more neutrophils and effectively reinforcing the cycle of inflammation.

Based on clinical studies and analyses of murine models of AP, our knowledge of the inflammatory events in AP can be summarized as: Damage to the pancreas, either caused by, and/or resulting in pancreatic autodigestion, leads to the release of inflammatory mediators from acinar cells. Many of these mediators are potent neutrophil attractants and are likely responsible for neutrophil recruitment to the pancreas as the first wave of inflammatory response. Once in the pancreas, activated neutrophils secrete additional cytokines, thus amplifying the inflammatory response by recruiting additional neutrophils as well as macrophages and other cells of the innate immune response. These cell types are often able to resolve the damage to the pancreas, with limited activation of the adaptive immune response. However, recurrent bouts of AP can lead to a much more serious condition, CP, which presents as a significant risk factor for the development of pancreatic cancer.

Whereas AP is often a self-limiting condition, CP is defined as longstanding inflammation of the pancreas that leads to progressive and irreversible changes. Clinically, CP is characterized by macrophage and T- and B-lymphocyte infiltration into the pancreas[118-122], although peripheral T-lymphocytes appear to decrease[123,124]. Infiltrating mast cells have also been described in the pancreas of CP patients[120,125,126], positively associating with pancreatic fibrosis[126] and pain[120,125]. Finally, immunosuppressive Tregs have been identified in the bone marrow, blood and lesions of CP patients[127].

In general, animal models of CP are generated by repeated induction of AP[128]. In a rat model of CP, both macrophages and $CD8^+$ T-lymphocytes were prevalent in the connective tissue and parenchyma, and $CD8^+$ T cells infiltrated the pancreatic lobules[118]. In a mouse model of CP, a significant increase in both macrophages and neutrophils were observed in response to caerulein (Figure 4), and depletion of these inflammatory cell types significantly reduced pancreatic injury as determined by serum amylase release, and pancreatic lesion formation and fibrosis[129,130]. Similar to clinical findings, mast cells were found to play a significant role in the pathogenesis of pain in a mouse model of CP[125]. Although a serious disease on its own, CP is also a significant risk factor for PDAC and may represent a condition that promotes PDAC development[131].

PANCREATIC CANCER

PDAC is the most common form of pancreatic cancer. PDAC is thought to develop by progression through a distinct series of pre-cancerous stages, pancreatic intraepithelial neoplasias (PanINs), before advancing to adenocarcinoma[132]. Greater than 90% of all pancreatic adenocarcinomas contain an activating mutation in codon 12 of the Kirsten rat sarcoma viral oncogene homolog (Kras)[133]. This mutation is thought to occur early in

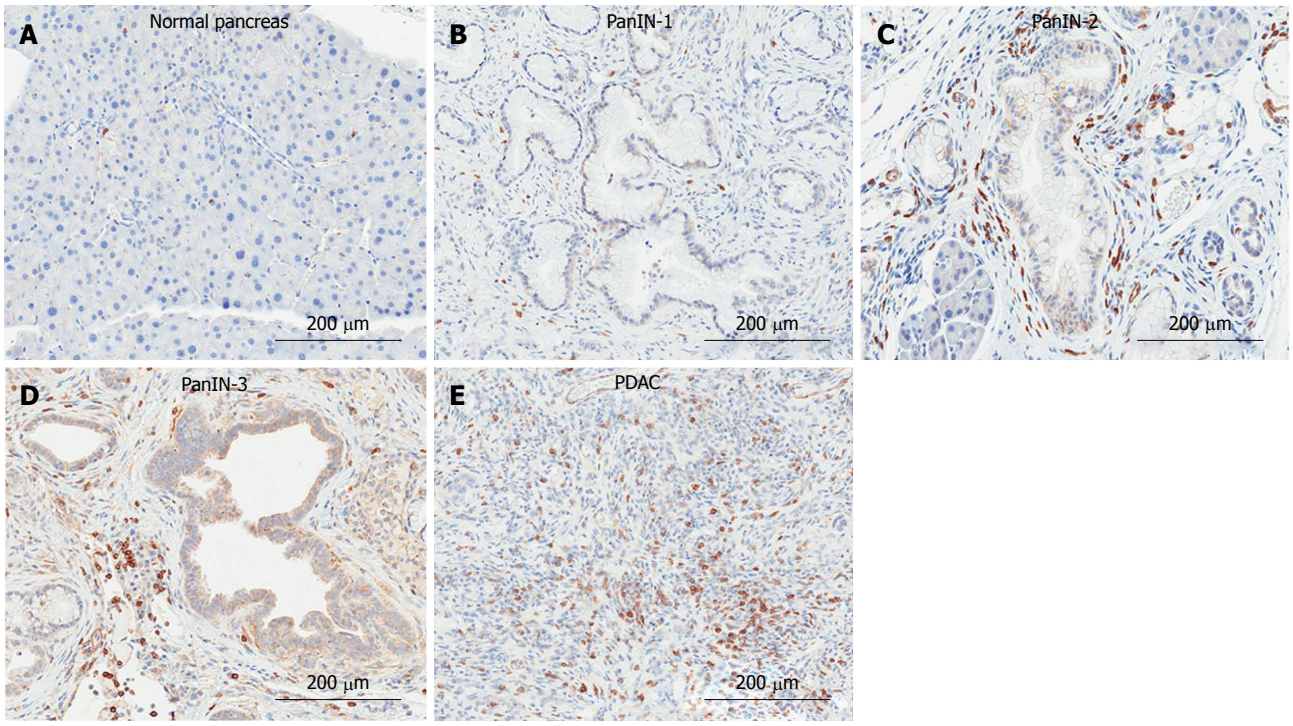

Figure 5 Immunohistochemical detection of T-lymphocytes in the Kras^{G12D}-induced mouse model of pancreatic ductal adenocarcinoma. A: Normal pancreas; B: Pancreatic intraepithelial neoplasia (PanIN)-1; C: PanIN-2; D: PanIN-3; E: Pancreatic ductal adenocarcinoma (PDAC) tissue was analyzed by immunohistochemistry for the presence of T-lymphocytes (CD3: abcam).

the disease process and drive initiation and progression of PDAC. Indeed, Kras mutations are prevalent even in early stage PanINs and their presence correlates with disease progression[134]. In a classic PDAC mouse model, tissue specific expression of oncogenic Kras^{G12D} from the endogenous Kras promoter (Lox-Stop-Lox- Kras^{G12D}; Pdx-1-cre, KC mouse model) in the mouse pancreatic epithelium recapitulates the full spectrum of human PDAC, including development of PanINs, with progression to adenocarcinoma at approximately 1-2 years of age[135]. For this reason, Kras^{G12D}-expressing mouse models are commonly used to study the initiation and development of PDAC.

It is well established that inflammation plays a major role in the development and progression of pancreatic cancer. Chronic inflammation of the pancreas (pancreatitis) is a significant risk factor for development of PDAC, and PDAC itself is characterized by marked leukocyte infiltration[4,47,48]. Notably, multiple immunosuppressive cell types are observed in pancreatic cancer tissue, suggesting a dysfunction of the immune response, likely mediated by the cancer itself (as described below). Immune dysfunction in PDAC is typified by (1) the recruitment and activation of immunosuppressive cell types; (2) the presence of tumor-supportive immune cells; and (3) a lack of immunity due to defective or absent immune cells. Clinical data and experimental rodent models contribute to the current understanding of the inflammatory response to PDAC as well as the impairment of that response that is a common evasion tool of most cancers.

Immunosuppression in PDAC

Clinical and murine evidence support the existence of profound immunosuppression in PDAC tissues. Clark *et al*[6] characterized the immune cell influx in various stages of PDAC ranging from normal pancreas to PanINs to invasive carcinoma using the KC mouse model of pancreatic cancer. The study describes an early immunosuppressive phenotype in pancreatic cancer, challenging the classic immunoediting "elimination" phase and suggesting that tumor cells "escape" from immune control almost immediately. At the early PanIN stages, Tregs and MDSCs dominate the immune infiltrate. As the disease progresses to PDAC, CD4$^+$ and CD8$^+$ cells are inconsistently found associated with the tumor, and those CD8$^+$ cells associated with the tumor lack evidence of activation, suggesting a suppressed immune environment[6]. In all stages of disease, there is a strong inverse correlation between MDSCs and CD8$^+$ T-lymphocytes, suggesting that MDSCs are a mediator of tumor immunosuppression[6].

Clinically, lymphocytes are prevalent in pancreatic cancer. CD8$^+$ cells are elevated in the circulation of PDAC patients[136], and leukocytes, the majority of which are T-lymphocytes, surround the pancreatic lesion[137]. T-lymphocytes are found more frequently in the fibrotic interstitial tissue than in the intraepithelial area of the pancreatic cancer[138], and distribute heterogeneously within the tissue, presenting as both scattered cells and focal areas of high accumulation[139]. An example of T-lymphocyte accumulation in progressive stages of the KC model of PDAC is shown in Figure 5.

The majority of the T-lymphocytes in PDAC are

$CD4^+$ Tregs, supporting an immunosuppressive phenotype. Tregs are significantly increased in the blood of PDAC patients as well as in the pancreatic tissue[140]. They are found typically in the stromal areas of the tumor, and only occasionally in association with tumor epithelial cells[141]. Hiraoka *et al*[141] examined clinical samples of pre-malignant lesions and found that Treg accumulation correlates with the progression of both of the major preneoplastic lesions in pancreatic cancer, PanINs and intraductal papillary mucinous neoplasms (IPMN). The association of Tregs with IPMN progression has been independently confirmed[142]. Additionally, Tregs correlate with metastasis[143] and tumor grade, and negatively correlate with patient survival[141].

Treg infiltration in the development of pancreatic cancer is confirmed in murine models of PDAC. A significant accumulation of Tregs is found in the KC model of PDAC[6]. In another murine tumor model, subcutaneous injection of mouse pancreatic tumor cells into syngeneic mice results in a significant increase in Tregs in the spleen and tumor-draining lymph nodes of these mice[144]. Tan *et al*[145] have shown in both human PDAC and a murine model of pancreatic cancer that tumor cells produce elevated levels of ligands for the CCR5 receptor, a receptor preferentially expressed by Tregs. Interruption of this receptor-ligand signaling reduces tumor size, as well as Treg recruitment to that tumor, suggesting Tregs are likely recruited in response to direct signaling from the tumor cells.

MDSCs are another immunosuppressive cell type prevalent in PDAC that contributes to immune dysfunction. Whereas MDSCs are absent in the healthy human pancreas, they are readily detected in the stroma of PDAC, comprising approximately 67% of the infiltrating leukocytes[146]. MDSCs are also found in the blood and bone marrow of PDAC patients, and are significantly higher in cases of metastatic disease compared to patients with local tumor[146-148], supporting a previous report that MDSC count correlates with disease stage[149]. Additionally, circulating MDSC numbers are found to be an independent prognostic factor for survival[147].

The functional role of MDSCs in tumor promotion has been characterized in murine models of PDAC[146,147,150]. Following subcutaneous injection of the non-metastatic pancreatic cancer cell line, Pan02, MDSC infiltration is detected in bone marrow, spleen, and tumor[146]. This increase is associated with decreased levels of CD4 and CD8 and increased levels of Tregs in circulation. These MDSCs are able to suppress $CD8^+$ T-lymphocytes *in vitro* and promote initial tumor growth when co-injected with Pan02[146]. Similarly, in a spontaneous murine model of tumor formation [driven by pancreas-specific overexpression of transforming growth factor (TGF) α and loss of Trp53], MDSC numbers increase in the lymph nodes, blood and pancreas as early as the pre-malignant lesion stage, and increase further upon tumor development. *In vitro*, these tumor-associated MDSCs were shown to possess arginase activity and suppress T-lymphocyte responses[151]. In the KPC model of metastatic pancreatic cancer (driven by pancreas-specific expression of oncogenic $Kras^{G12D}$ and mutant $Trp53^{R172H}$), MDSCs accumulate in tumor and spleen and comprise 20%-30% of all leukocytes. MDSCs are closely associated with tumor cells and metastases, suppress proliferation of T-lymphocytes, and express high levels of arginase and nitrite upon stimulation[150]. Collectively, these animal models strongly support a role for MDSCs in tumor promotion through T-lymphocyte inhibition and resulting immunosuppression.

To summarize, both clinical and animal models provide strong evidence for accumulation of immunosuppressive cells types, and subsequent inhibition of immune function in PDAC. The consequence of a suppressed immune system is not direct tumor promotion, but rather alleviation of an important barrier to tumor growth. As described by the cancer immunoediting model, a functional immune system can act as an extrinsic tumor suppressor, identifying and eliminating tumor cells[10]. Alterations in tumor immunogenicity or suppression of the immune response are crucial mechanisms by which this barrier is surmounted, allowing the cancer to progress.

Tumor-supportive immune cells

In addition to the immunosuppressive cell types that inhibit effector T-lymphocyte immunity, other immune cells adopt a tumor-supportive role in the context of PDAC. Once a tumor is established, factors secreted by effector immune cells can often promote, rather than prevent, tumor progression. Mast cells accumulate in PDAC tissue and, along with macrophages, express tumor-promoting factors including vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF)[152]. VEGF and FGF have been shown to stimulate the growth of pancreatic tumors and maintain blood vessels[153,154]. Mast cell accumulation correlates with higher tumor grade, worse survival[155], lymphatic and microvascular invasion[156], and lymph node metastasis[152,156]. In support of the idea that mast cells can support tumor growth, *in vitro* analyses demonstrate that mast cell-derived factors can promote PDAC cell migration, proliferation, and invasion[155,157]. Interestingly, the importance of mast cells in PDAC appears to be zone specific, with the presence of mast cells in the intratumoral border zone, and not in the intratumoral center or peritumoral zones, correlating with microvessel proliferation, lymph node metastases, and lymphatic and microvascular invasion[156]. Additionally, mast cell accumulation within the intratumoral border is an independent prognostic factor for survival[156]. The significance of mast cell localization likely stems from the ability to establish a pro-tumor microenvironment by degrading tissue surrounding the tumor to promote invasion and by remodeling blood vessels[156]. Taken together, preclinical PDAC models support a role for mast cells in tumor growth and for mast cell accumulation and function at the tumor border[158].

Inflammation can drive the early stages of pancreatic

lesion formation in mouse models[159]. A recent study demonstrated that two macrophage secreted inflammatory cytokines could mediate the effects of infiltrating macrophage on acinar cell metaplasia[5]. Matrix metalloproteinase (MMP)-9 and RANTES (regulated on activation, normal T cell expressed and secreted), secreted by pancreas-infiltrating macrophages, are implicated in driving the very first stages of pancreatic lesion formation in a mouse model of cearulein-induced pancreatitis[5].

Immune factors that are responsible for pathogen killing can also promote tumor cell migration and metastasis[160]. Additionally, tumor-promoting immune factors can promote tumor cells to adopt a more fibroblast-like morphology[155,157,160-162] and remodel the tumor environment[156,163] to facilitate migration. In these ways, neutrophil- and B-lymphocyte-derived proteins are implicated in PDAC invasion[160-163]. Neutrophil-derived elastase has been shown to mediate epithelial-mesenchymal transition (EMT) of PDAC cells *in vitro*[161,162]. Neutrophil-derived MMP-9 promotes PDAC tumor growth and angiogenesis[163], and MMP-9-producing neutrophils have been identified at the leading edge of PDAC in a murine model of pancreatic cancer as well at the invasive front of PDAC metastases[164]. Neutrophils, typically one of the body's first lines of defense, are not prevalent in PDAC epithelia[165]. However, they are associated with the predominant stromal component of PDAC, and several reports describe a significant increase in neutrophils when measuring levels in both PDAC tissue and stroma together[162,166]. In PDAC, neutrophils are associated with micropapillary carcinoma of the pancreas and correlate with poor prognosis[165,167]. Although density of neutrophil infiltrate does not correlate with tumor clinical stage[162], a patient's neutrophil: lymphocyte ratio is an independent prognostic indicator of survival following resection of PDAC tumor[168,169].

B-lymphocytes are also able to promote the tumor phenotype by secretion of B-lymphocyte activating factor (BAFF). Koizumi *et al*[160] described *in vitro* data demonstrating that BAFF mediates EMT, increases invasion and promotes motility of PDAC cells, supporting a role for B-lymphocyte-secreted BAFF tumor progression and metastasis. Additionally, BAFF-expressing B-lymphocytes surround and infiltrate tumor cells in clinical samples of PDAC, correlating with increased serum levels of BAFF[160].

Defective immune cells in PDAC

DCs are the link between innate and adaptive immunity, recognizing the presence of foreign pathogens and alerting the adaptive immune cells *via* antigen presentation. However, in a tumor environment, DCs often display an immature phenotype and are defective in their antigen-presenting abilities[170-173]. Tumors express various molecules which are thought to repress DC maturation[174] including IL-10[175,176], IL-4[177], VEGF[178], TGF-β[179], IL-6[180,181], and macrophage-colony stimulating factor[181]. Muc-1, a protein highly expressed on PanIN cells profoundly affects the maturation of DCs. Monti *et al*[182] demonstrated that DCs exposed to tumor mucins do not fully mature and are characterized by a tolerogenic/regulatory cytokine profile, expressing high levels of IL-10 and low levels of IL-12. Peripherally, circulating DCs are significantly decreased in patients with PDAC[183-186], and demonstrate an impaired ability to stimulate T-lymphocyte proliferation[184].

Absent immune cells in PDAC

Similar to chronic pancreatitis, few NKCs are found in PDAC tissue[138]. The basal systemic NKC activity is decreased in PDAC patients, as well as the NKC response to interferon-α, a potent enhancer of NKC activity[187,188]. Decreased NKC activity has been linked to poor patient prognosis[188] and may be indicative of a suppressed innate immune response.

A role for epithelial cells in dysfunction of the immune response

Several lines of evidence point to a strong role for the neoplastic pancreatic epithelial cells in establishing a dysfunctional immune environment. As previously mentioned, PDAC cells can recruit Tregs[145], promote mast cell migration and activation[157], and repress DC maturation[182]. *In vitro*, human PDAC cells inhibit T-lymphocyte proliferation and migration, and this is accompanied by an increase in immunosuppressive cytokines including IL-8 and TGFβ[189]. Immunosuppressive compounds TGFβ and IL-10 are upregulated in PDAC and pancreatitis patient sera[190]. Finally, Muc-1, a mucin highly expressed in PanIN-1 early lesions, can suppress T-lymphocyte proliferation[191,192] and it has been suggested that Muc-1 is responsible for tumor escape from recognition and destruction by immune cells[193].

Murine models of PDAC provide additional support for the role of pancreatic epithelial cells in maintaining an immunosuppressive tumor environment. In the KC mouse model, $Kras^{G12D}$-dependent upregulation of granulocyte macrophage-colony stimulating factor (GM-CSF) is detected in PanINs, and results in recruitment of MDSCs and concomitant inhibition of $CD8^+$ T-lymphocytes[194]. This data is supported by the work of Bayne *et al*[150] who describe a model in which tumor-derived GM-CSF recruits myeloid inflammatory cells resulting in the negative regulation of $CD8^+$ T cells.

Interaction(s) between immune cells and stroma in PDAC

The development of PDAC is marked by increasing desmoplasia, resulting in the development of a vast stroma that often equals or exceeds the epithelial component of the tumor. The stroma is composed of extracellular matrix proteins and contains various non-epithelial cell types including stellate cells, endothelial cells, and immune cells, but the majority of the stromal cells are activated pancreatic stellate cells (PSCs) and fibroblasts[195]. Activated PSCs promote cancer cell growth and immune cell dysfunction

in PDAC[196,197]. PSCs have been implicated in promoting transformed growth, cellular invasion and EMT of PDAC cells, as well as in promoting PDAC tumor incidence, size, and metastasis in an orthotopic mouse model[198-200].

PSCs can have profound effects on the immune cell milieu of PDAC, and have been shown to express a number of growth factors and cytokines[201]. Inflammatory cells recruited to the tumor site are most often found in the stroma rather than infiltrating the epithelial cells[202]. Indeed, activated PSCs have been shown to attract and adhere to $CD8^+$ T-lymphocytes, sequestering them in a juxtatumoral compartment (< 100 μm from tumor) and preventing their access to PDAC tumor cells[197]. One report showed that 94% of tumor-associated T-lymphocytes were either inactivated or did not make it to tumor because they were trapped in the tumor stroma[190]. A potential mechanism by which PSCs may regulate T-lymphocyte trafficking in PDAC is *via* CXCL12 expression, as PDAC T-lymphocytes express elevated levels of the CXCL12 receptor[197]. Additionally, activated PSCs express a number of cytokines and adhesion-mediating molecules[203], and have been shown to produce MDSC-promoting cytokines, including IL-6, M-CSF, and VEGF, and to promote differentiation of MDSCs from peripheral blood mononuclear cells[204]. Finally, PSCs stimulate mast cell activation, and, conversely, mast cell-derived factors can stimulate PSC proliferation[157]. These data suggest that not only can activated stellate cells directly promote the cancer cell phenotype, but they also contribute to the immunosuppressive phenotype that characterizes PDAC and hampers immunotherapy efforts.

Cancer stem cells

In addition to the immune dysfunction that is prevalent in PDAC, new evidence is emerging to support a role for immune-cytokines in promoting cancer metastasis *via* interaction with cancer stem cells (CSCs). CSCs are characterized by their ability to self-renew, capability to develop into multiple lineages, enhanced tumor-initiating ability, and resistance to typical cancer therapies[205]. CSCs are thought to be responsible for cancer progression, resistance to standard therapies and tumor relapse. Specifically, pancreatic CSCs have been shown to play a crucial role in the aggressive nature of pancreatic cancer and the resistance to therapy that is a hallmark of this cancer (reviewed in Dorado *et al*[206]). They share an intimate relationship with the tumor microenvironment, regulating, and being regulated by, cells present therein[207,208]. Specifically, TAMs regulate CSC tumorigenicity and anticancer drug resistance through production of specific growth factors[208]. Additionally, inflammatory cytokines play a role in mediating CSC self-renewal[209].

Recent work suggests that inflammatory cells can promote dissemination of pancreatic CSCs. Rhim *et al*[210] reported that pancreatic cancer cells exhibiting cancer stem cell properties, including enhanced tumor-initiating capacity, survival, and self-renewal, left the pancreas and were detected in the circulation at the immediate early stages of pancreatic cancer development (PanIN formation), prior to overt tumor formation. The presence of CSCs in the circulation was significantly elevated following induction of pancreatitis, and conversely, treatment with an anti-inflammatory drug, dexamethasone, resulted in a significant decrease in the circulating CSCs. These data support a role for inflammation in promoting dissemination of pancreatic CSCs and potentially in PDAC metastasis.

THERAPY FOR PDAC

Immunotherapy, therapeutic modulation of the immune response, has emerged as a promising line of treatment for many cancers including PDAC[211,212]. Types of immunotherapy that are currently being tested in clinical trials for pancreatic cancer include whole cell, peptide/DNA, antigen pulsed DC, and monoclonal antibody vaccines. Whole cell vaccines typically use irradiated pancreatic cancer cells as the immunogen. These cells have the potential to elicit a robust immune response because they express the full complement of tumor-associated antigens. There have been significant survival advantages reported in resected pancreatic cancer patients using whole cell vaccines such as Algenpantucel-L, an irradiated, live combination of two allogenic pancreatic cell lines[213]. Vaccines comprised of peptides or DNA corresponding to tumor antigens are designed to enhance the cytotoxic T-lymphocyte response. Peptides corresponding to oncogenic Ras, telomerase, VEG-F receptor, carcinoembryonic antigen (CEA), survivin, and Muc-1 have all been successful at prolonging life in pancreatic cancer patients in clinical trials[211,213].

Antigen-pulsed DC vaccines take advantage of the antigen-presenting abilities of DCs to elicit a robust adaptive immune response specific to a tumor antigen of choice[214]. DCs pulsed with Muc-1 and CEA antigens have both been used in clinical trials for pancreatic cancer[215]. Finally, monoclonal antibodies against cell surface tumor antigens are used to induce antibody-dependent cell cytotoxicity[211]. Clinical trials have evaluated antibodies against mesothelin, CEA, and epidermal growth factor receptor for pancreatic cancer treatment[211,215]. Of importance, the success of an anti-cancer vaccine relies on its ability to elicit an immune response in the host, and thus may not exhibit uniform effectiveness in all patients depending on the ability of their immune system to generate a response to treatment.

The stromal compartment has drawn recent interest as a target for PDAC therapy. The stroma creates an inflammatory environment and promotes tumor progression; and the extent of activated stroma has been identified as a novel independent prognostic marker in PDAC[216]. Several potential PDAC therapies have targeted the stroma[217-219]. One method in particular aims at overcoming the immunosuppression often found in, and potentially caused by, cells of the stroma. CD40 agonists take advantage of the inflammatory cells found within the stroma[218].

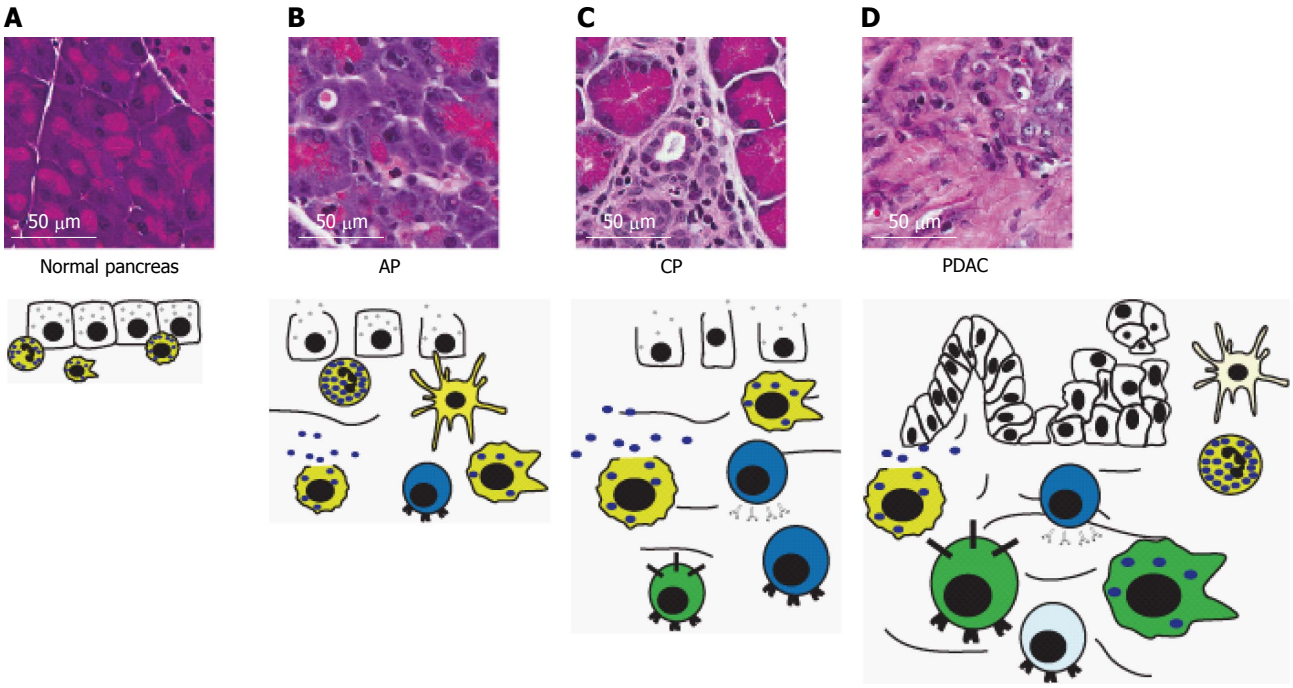

Figure 6 Immune cells in progressive pancreatic disease. A: The normal pancreas contains sparse, mostly innate, inflammatory cells and lacks the dense stroma typically seen in chronic pancreatitis (CP) and pancreatic ductal adenocarcinoma (PDAC); B: Acute pancreatitis (AP) is characterized by acinar degranulation, edema and recruitment of mostly innate inflammatory cells, but also some T-lymphocytes in response to acinar damage. Pancreatic mast cells begin to degranulate; C: CP is characterized by development of stroma surrounding degranulated acinar cells, acinar-to-ductal metaplasia and edema. Additionally, there is an increased presence of macrophages, and T- and B-lymphocytes, and further degranulation of mast cells; D: Development of pancreatic intraepithelial neoplasias and subsequent PDAC leads to a significant increase in immunosuppressive cell types including tumor-associated macrophages, myeloid-derived suppressor cells, and Tregs. Degranulated mast cells, neutrophils, dendritic cells and B- and T-lymphocytes are also present. However, T-lymphocytes and dendritic cells are typically inhibited and defective, respectively. Size of immune cell represents relative abundance. Cell color denotes immune cell type: yellow, innate immune cells; blue, adaptive immune cells; green, immunosuppressive cells. For description of graphical representation of cell types, see Figure 3 legend. Cells that are inactivated or defective are represented by a lighter color.

CD40 is the co-stimulatory factor needed for activation of the T-lymphocyte-dependent anti-tumor response of the immune system. It is thought that activating stromal T-lymphocytes may overcome the immunosuppressive environment that is a hallmark of PDAC. Co-treatment with a CD40 agonist and gemcitabine showed therapeutic efficacy in patients with metastatic PDA[218].

Whereas cancer immunotherapy has typically involved treatment with cancer antigens to stimulate or boost the anti-cancer immune response, the immunosuppressive environment found in PDAC presents obvious challenges. Vaccination with self-antigens has been associated with induction of immunosuppressive cell types, thus potentiating, rather than inhibiting, tumor growth[220]. Successfully overcoming the immunosuppressive environment that characterizes PDAC will likely require a multifaceted approach due to the multiple mechanisms by which tumor-associated immune dysfunction seems to occur. However, new evidence suggests that immunotherapy can be successful for pancreatic cancer, if stimulation of the immune system is combined with control over the immunosuppressive environment[221,222]. Developing new methods to overcome immunosuppression or exploit the immune response to target PDAC may be utilized in combination with conventional or novel chemotherapy to enhance the survival of this currently deadly disease.

CONCLUSION

The immune system protects the host from invading pathogens and foreign materials. The aberrant expression profile and uncontrollable proliferation that characterizes tumor cells should allow for recognition as non-self by the immune system. However, we now know that from a very early stage in PDAC development, the ability of the immune system to identify and eliminate neoplastic cells is compromised. This suggests that an immunosuppressive environment is established early in tumor development to effectively thwart the immune response to neoplastic cells at the onset of tumor development. Figure 6 depicts the progressive changes in immune cell infiltrate found during various stages of pancreatic disease.

Based on data reviewed here, neoplastic cells produce compounds (such as GM-CSF) at a very early stage of pancreatic cancer development, that recruit immunosuppressive immune cells, potentially facilitating progression to later PanIN stages and PDAC[190-192,194]. Once PDAC cells are present, they actively prevent the maturation of dendritic cells, inhibiting their antigen presenting activity and effectively cutting off a major communication between the innate and adaptive immune response[182]. In addition, immunosuppressive Tregs and MDSCs accumulate in the blood, stroma and PDAC tissue and inhibit T-lym-

phocyte proliferation[6,140,141,144-146]. In this setting, even immune cell types considered pro-inflammatory add to the tumor-supportive environment: neutrophils accumulate in the stroma and secrete proteases that aid in EMT, tumor motility and invasion[161,163] and mast cells accumulate in the tumor tissue and express factors used by the tumor to sustain its growth[152]. It is clear that a complex relationship exists between the immune system and the developing pancreatic cancer, and that these interactions have important implications for disease prevention and control. Immunotherapy can potentially be a powerful component of PDAC treatment. Further study of the mechanisms by which immunosuppression is initiated in PDAC, and ways to overcome it, will facilitate the development of this treatment option[22,104,223-249].

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P- Reviewer: Ghiorzo P, Schuurman HJ, Tanaka S, Zhong QY
S- Editor: Zhai HH **L- Editor**: A **E- Editor**: Wang CH

World Journal of Gastroenterology

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DOI: 10.3748/wjg.v20.i32.11182

World J Gastroenterol 2014 August 28; 20(32): 11182-11198
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (14): Pancreatic cancer

Pancreatic ductal adenocarcinoma: Risk factors, screening, and early detection

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Received: November 1, 2013 Revised: February 15, 2014
Accepted: April 15, 2014
Published online: August 28, 2014

Abstract

Pancreatic cancer is the fourth most common cause of cancer-related deaths in the United States, with over 38000 deaths in 2013. The opportunity to detect pancreatic cancer while it is still curable is dependent on our ability to identify and screen high-risk populations before their symptoms arise. Risk factors for developing pancreatic cancer include multiple genetic syndromes as well as modifiable risk factors. Genetic conditions include hereditary breast and ovarian cancer syndrome, Lynch Syndrome, familial adenomatous polyposis, Peutz-Jeghers Syndrome, familial atypical multiple mole melanoma syndrome, hereditary pancreatitis, cystic fibrosis, and ataxia-telangiectasia; having a genetic predisposition can raise the risk of developing pancreatic cancer up to 132-fold over the general population. Modifiable risk factors, which include tobacco exposure, alcohol use, chronic pancreatitis, diet, obesity, diabetes mellitus, as well as certain abdominal surgeries and infections, have also been shown to increase the risk of pancreatic cancer development. Several large-volume centers have initiated such screening protocols, and consensus-based guidelines for screening high-risk groups have recently been published. The focus of this review will be both the genetic and modifiable risk factors implicated in pancreatic cancer, as well as a review of screening strategies and their diagnostic yields.

Key words: Pancreatic neoplasms; Pancreas cancer screening; Genetic predisposition to disease; Hereditary breast and ovarian cancer syndrome; Lynch syndrome; Peutz-Jeghers; *BRCA*; *PALB2*; *p16*; Pancreatitis

Core tip: Risk factors for developing pancreatic cancer include multiple genetic syndromes as well as modifiable risk factors. These factors can raise the risk of developing pancreatic cancer up to 132-fold over the general population. Several large-volume centers have initiated screening protocols, and consensus-based guidelines for screening high-risk groups have recently been published. The focus of this review will be both the genetic and modifiable risk factors implicated in pancreatic cancer, as well as a review of screening strategies and their diagnostic yields.

Becker AE, Hernandez YG, Frucht H, Lucas AL. Pancreatic ductal adenocarcinoma: Risk factors, screening, and early detection. *World J Gastroenterol* 2014; 20(32): 11182-11198 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11182.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11182

INTRODUCTION

Pancreatic cancer is the fourth most common cause of cancer-related deaths in the United States, with an estimated over 45000 diagnoses and 38000 deaths in 2013[1]. Pancreatic ductal adenocarcinomas (PDAC) arise from the exocrine pancreas and account for 95% of pancreatic cancers. The lifetime risk of developing pancreatic cancer

Table 1 Selected pancreatic ductal adenocarcinoma genetic risk factors

Risk factor	Gene	Increased PDAC risk	Other associated cancers
Hereditary breast and ovarian cancer syndrome	*BRCA1, BRCA2, PALB2*	2-3.5	Breast, ovarian, prostate
Lynch syndrome (hereditary non-polyposis colorectal cancer)	*MLH1, MSH2, MSH6, PMS2, EPCAM*	8.6	Colon, endometrium, ovary, stomach, small intestine, urinary tract, brain, cutaneous sebaceous glands
Familial adenomatous polyposis	*APC*	4.5-6	Colon, desmoid, duodenum, thyroid, brain, ampullary, hepatoblastoma
Peutz-Jeghers syndrome	*STK11/LKB1*	132	Esophagus, stomach, small intestine, colon, lung, breast, uterus, ovary
Familial atypical multiple mole melanoma pancreatic carcinoma syndrome	*P16INK4A/CDKN2A*	47	Melanoma
Hereditary pancreatitis	*PRSS1, SPINK1*	69	
Cystic fibrosis	*CFTR*	3.5	
Ataxia-telangiectasia	*ATM*	Increased	Leukemia, lymphoma
Non-O blood group		1.3	
Familial pancreatic cancer	Unknown	9 (1 FDR) 32 (3 FDRs)	

PDAC: Pancreatic ductal adenocarcinomas; FDR: First-degree relative.

Table 2 Selected pancreatic ductal adenocarcinoma modifiable risk factors

Risk factor	Increased PDAC risk
Current cigarette use	1.7-2.2
Current pipe or cigar use	1.5
> 3 alcoholic drinks per day	1.2-1.4
Chronic pancreatitis	13.3
BMI > 40 kg/m^2, male	1.5
BMI > 40 kg/m^2, female	2.8
Diabetes mellitus, type 1	2.0
Diabetes mellitus, type 2	1.8
Cholecystectomy	1.2
Gastrectomy	1.5
Helicobacter pylori infection	1.4

PDAC: Pancreatic ductal adenocarcinomas; BMI: Body mass index.

is 1.49%, or 1 in 67, with incidence increasing with age[2]. Epidemiologically, the incidence rates of PDAC are higher in males, African Americans, and lower socioeconomic status groups[1].

Both genetic and modifiable risk factors contribute to the development of PDAC. A hereditary component has been identified in approximately 10% of cases, with a specific germline mutation being implicated in 20% of those cases[3,4]. These genetic conditions, including the hereditary breast and ovarian cancer syndrome (HBOC), Lynch syndrome (HNPCC), familial adenomatous polyposis (FAP), Peutz-Jeghers syndrome (PJS), familial atypical multiple mole melanoma syndrome (FAMMM), hereditary pancreatitis (HP), cystic fibrosis (CF), and ataxia-telangiectasia (AT), have been shown to raise the risk of PDAC anywhere from 2 to 132-fold (Table 1)[5-7]. Modifiable risk factors, which include tobacco exposure, alcohol use, chronic pancreatitis, diet, obesity, diabetes mellitus, as well as certain abdominal surgeries and infections have also been identified as increasing the risk of PDAC (Table 2).

PDAC is nearly universally lethal: less than 20% of patients are surgical candidates at the time of presentation, and the median survival for non-resected patients is 3.5 mo[8]. Even among those patients who are candidates to undergo pancreatectomy, the median survival is 12.6 mo[8]. However, by identifying and screening patients at an increased risk of developing PDAC, detection of precursor and early-stage lesions may allow diagnosis at a still surgically-resectable stage. Several large-volume centers have initiated screening protocols, and consensus-based guidelines for screening high-risk groups have recently been published[3,9]. The focus of this review will be both the genetic and non-genetic risk factors implicated in PDAC, as well as screening strategies and their diagnostic yields.

PDAC RISK FACTORS

PDAC risk factors: Genetic

It has been reported that up to 10% of PDAC have a hereditary component[4]. A 2009 meta-analysis demonstrated that having just one affected relative resulted in an 80% increased risk of developing PDAC[10]. Specific mutations in multiple genes have been implicated in causing roughly 10% of PDAC, with varying penetrance and degree of increased cancer risk for each mutation (Table 1)[11,12]. Identification and stratification of individuals at increased risk of having genetic mutations may allow for a group of patients that will benefit from early detection of these pancreatic neoplasms, as well as targeted, gene-specific therapy.

Hereditary breast and ovarian cancer syndrome and other fanconi anemia genes: *BRCA1*, *BRCA2/FANCD1*, *PALB2/FANCN*, *FANCC*, and *FANCG*: Fanconi anemia is an autosomal recessive disease characterized by multiple congenital anomalies, bone-marrow failure, and increased susceptibility to malignancy, including acute myeloid leukemia and head and neck squamous cell carcinoma[13,14]. There are 15 Fanconi anemia genes, and products of these genes are involved in multiple DNA repair

mechanisms, including the *BRCA1/2* pathway[13,14]. The incidence of the disease is 1 in 100000 live births, and the carrier rate of Fanconi anemia mutations is estimated at 1 in 300[13,15].

HBOC is characterized by early-onset breast and ovarian cancers resulting from monoallelic germline mutations in the *BRCA1* or *BRCA2* (also known as *FANCD1*) genes. These tumor suppressor genes code for proteins that repair double-stranded DNA breaks. While *BRCA2* codes for a Fanconi anemia protein, the *BRCA1* protein directly interacts with the FANCA protein[16]. *BRCA1/2* mutations have been shown to have a population frequency of 1.0%, with a higher concentration within the Ashkenazi Jewish population (2.3%)[17,18]. These genes have high penetrance with respect to female breast cancer (cumulative risk by age 70 of 57% for *BRCA1* and 49% for *BRCA2*) and ovarian cancer (cumulative risk by age 70 of 40% for *BRCA1* and 18% for *BRCA2*), and lower rates for male breast cancer (cumulative risk by age 70 of 1.2% for *BRCA1* and 6.8% for *BRCA2*) as well as PDAC[19]. While a few large studies have indicated that *BRCA1* mutations are associated with a roughly 2-fold increased risk of PDAC, the mutation is rarely seen in PDAC families without a strong history of breast cancer[6,7,20]. Additionally, not all studies have found an increased risk of PDAC among the *BRCA1* cohort[21]. On the other hand, the evidence for an association between *BRCA2* germline mutations and PDAC is more clearly defined. With a relative risk of at least 3.5, *BRCA2* mutations have been identified as the most common known inherited cause of PDAC: studies have found deleterious mutations in the *BRCA2* gene in 17%-19% of familial pancreatic cancer families and 7.3% of apparently sporadic pancreatic cancers[22-25]. Our group has demonstrated an increased prevalence of *BRCA1* mutations (8.3%) and *BRCA2* mutations (10.8%) in a cohort of unselected Ashkenazi Jewish patients who underwent surgical resection for PDAC and IPMN; half of those *BRCA1/2*-associated tumors demonstrated loss of heterozygocity[26]. In a registry study of *BRCA1* and *BRCA2* families, there was a significantly earlier age of onset (age 63 for each) for PDAC, compared to that found in the SEER database (age 70)[27].

PALB2, or partner and localizer of *BRCA2* (also known as *FANCN*), is a gene that codes for a protein which stabilizes the *BRCA2* protein as it repairs DNA. *PALB2* is known to be a breast cancer susceptibility gene and has been found to be mutated in up to 3% of familial PDAC[28,29]. While some large registry cohort studies have not found *PALB2* mutations to increase the relative risk of PDAC, other groups have identified *PALB2* mutations in multiple familial pancreatic cancer families[30-33]. Additionally, it has been demonstrated that relatives of *PALB2* mutation carriers have a 6-fold increased risk of PDAC compared to relatives of those with the wild-type gene[34].

Mutations in two other Fanconi anemia proteins, specifically *FANCC* and *FANCG,* have shown loss of heterozygosity in young-onset (< 55 years of age) PDAC[35,36]. No studies to date have found an increased risk of PDAC associated with mutations in these genes.

Targeted therapy is a promising area of research for genes in this pathway. Cells deficient in *BRCA1, BRCA2/FANCD1*, *PALB2/FANCN*, *FANCC* or *FANCG* must use DNA repair mechanisms that are more error prone and resultant mutations are more likely to result in cell death. Thus, agents that induce DNA damage or inhibit other repair mechanisms may affect deficient cells more than fully-functional cells[37]. *In vitro* cells deficient in these proteins and *in vivo* cells in mice were shown to be hypersensitive to alkylating agents such as mitomycin C, cisplatin, chlorambucil, and melphalan, whereas normal cells were unaffected[38,39]. Additionally, poly (ADP-ribose) polymerase (PARP) inhibitors have been shown to have anti-tumor activity in multiple other human cancers[40]. There have been case reports of complete pathological response of *BRCA2*-associated PDAC to PARP inhibitors, and clinical trials are currently underway[41].

Lynch syndrome (or HNPCC): *MLH1*, *MSH2*, *MSH6*, *PMS2*, and *EPCAM*: Lynch syndrome, the most common inherited colorectal cancer syndrome, is characterized by early-onset colorectal cancer as well as a predisposition to cancer of the endometrium, ovary, stomach, small intestine, urinary tract, brain, pancreas and cutaneous sebaceous glands[42]. The incidence of this syndrome has been postulated to be between about 1:660 to 1:2000[43]. The *MSH2*, *MSH6*, *MLH1*, *PMS2*, and *EPCAM* genes, which are mutated in this syndrome, normally code for proteins involved in the DNA mismatch repair pathway which bind to mismatched double-stranded DNA and microsatellites to target and prepare them for repair[42]. Patients with Lynch syndrome have an 8.6-fold increased risk of developing PDAC compared to the general population[44]. These pancreatic tumors often have a characteristically medullary appearance, with prominent lymphocytic infiltration and microsatellite instability[44,45].

FAP: *APC*: FAP is characterized by the early development of hundreds to thousands of colorectal adenomatous polyps; some of these polyps inevitably progress to malignancy, conferring an almost 100% risk of colorectal cancer by age 40[46]. There is also an increased risk of extracolonic cancers including desmoid, duodenum, thyroid, brain, ampullary, pancreas, and hepatoblastoma tumors[47]. The incidence of FAP in the Northern European population is 1 in 13000-18000 live births in the Northern European population[48,49]. FAP is caused by a mutation in the *APC* gene, a tumor suppressor gene which codes for a scaffolding protein responsible for targeting β-catenin for destruction, as well as acting as a control on progression of the cell cycle and a microtubule stabilizer[47]. Specifically, the relative risk of PDAC in FAP is reported to be 4.5 to 6-fold, although it is uncertain if this represents a true increased risk of PDAC or reflects misclassification

of ampullary carcinomas[50,51]. There also exists a subset of the FAP population with an attenuated phenotype, known as attenuated FAP (AFAP) that is also caused by a mutation in the *APC* gene; this population has fewer colorectal adenomatous polyps (10-100) and a fifteen-year delay in the onset of colorectal cancer compared to those with FAP[52]. Compared to FAP, AFAP is associated with a lower risk of extracolonic cancers[53].

PJS: *STK11/LKB1*: PJS is characterized by hamartomatous gastrointestinal polyposis and distinctive mucocutaneous pigmentation found most commonly on the lips or perioral region[45,54]. PJS, with an estimated frequency of 1:8300 to 1:280000, is associated with an inherited mutation in the *STK11/LKB1* gene, a tumor suppressor gene which encodes for a serine/threonine kinase[45]. While the exact mechanism by which the *LKB1* gene acts as a tumor suppressor is unknown, PJS tumors have shown less activated AMP-kinase, which results in mammalian target of rapamycin hyperactivation[55]. Additionally, *LKB1* haploinsufficiency has been shown to cooperate with *K-ras* to cause PDAC in the mouse model, through a decrease in growth arrest[56]. A 2000 meta-analysis demonstrated that PJS is associated with a relative risk of 15.2 for all cancers and a 93% overall rate of cancer by age 64[5]. The study found a statistically significant increased risk of esophageal, stomach, small intestine, colon, pancreas, lung, breast, uterus, and ovarian cancers, including a relative risk of 132 for PDAC.

FAMMM: *p16INK4A/CDKN2A*: FAMMM is characterized by malignant melanoma in one or more first-degree relatives (FDRs) or second-degree relatives (SDRs) and multiple, atypical melanocystic nevi[55]. The prevalence of FAMMM is unknown. While there is variability in the underlying genetics of this syndrome, a germline mutation in the *p16INK4A* (also known as *CDKN2A* or *MTS1*) gene has been found in approximately 38% of the cases of this syndrome[57,58]. FAMMM with this particular mutation, which confers a 60%-90% risk of melanoma by age 80, is called FAMMM pancreatic carcinoma syndrome (FAMMM-PC) because those with the *p16INK4A* mutation have also demonstrated an increased risk of PDAC[59-62]. This gene, which codes for the *p16* protein, is a tumor suppressor gene involved in the regulation of cell cycle progression. A study following 19 FAMMM families over seventy years found a 13 to 22-fold increased risk of developing PDAC in those with this *p16INK4A* mutation; conversely, they found no cases of PDAC in those without this mutation[63]. More recently, a relative risk of PDAC of 47 was demonstrated among those with this *p16INK4A* mutation compared to the general population[64]. The risk of PDAC was even more apparent when looking at those under 55 years of age: a Swedish study found the relative risk to be 65-fold for *p16* mutation carriers[61].

HP and CF: *PRSS1*, *SPINK1* and *CFTR*: HP is characterized by recurrent attacks of acute pancreatitis starting in childhood, which can lead to pancreatic failure[65]. About 80% of HP is caused by a germline mutation in the *PRSS1* gene, which codes for the prodigestive enzyme trypsinogen[66]. Defective mutations result in either premature activation or reduced deactivation of the enzyme, leading to pancreatic injury. The *SPINK1* gene codes for a serine protease inhibitor that inhibits active trypsin; mutations in this gene have also been associated with various forms of pancreatic disease, including pancreatitis[67]. HP has an 80% penetrance rate[68]. A 2010 meta-analysis found a relative risk of 69 for PDAC for patients with HP compared to the general population[69].

Additionally, homozygous mutations in the autosomal recessive *CFTR* gene cause cystic fibrosis, which is associated with both a younger age of onset (median age of 35 years) and 5.3-fold greater risk of the development of PDAC[70]. However, even when a *CFTR* gene mutation is inherited in a heterozygous fashion, it has been demonstrated that this confers a 4-fold greater chance of developing chronic pancreatitis[66,71,72].

The presence of chronic inflammation in pancreatitis is thought to be the primary mechanism by which PDAC develops. A few mechanisms have been suggested as methods by which inflammation leads to PDAC[73]. Inflammatory cytokines such as IL-6 and IL-11 may induce the proliferation and facilitate survival of malignant and premalignant cells through the activation of multiple transcription factors, including STAT3 and NF-κB. Additionally, chronic inflammation may suppress immunosurveillance as well as inhibit oncogene-induced senescence, which would allow the lesion to develop unchecked. It has been suggested that increased activation of pancreatic stellate cells leads to fibrosis *via* increased cell proliferation and inflammation[74].

AT: *ATM*: AT is an autosomal recessive, progressive neurologic disorder characterized by early ataxia and later telangiectasias of the blood vessels on exposed areas of the skin and eyes, with cerebellar ataxia, varied immune dysfunction, an extreme sensitivity to ionizing radiation, and an increased risk of cancers, particularly leukemias and lymphomas[75-77]. The estimated incidence of AT is 1 in 40000-300000 live births, and the disease is caused by a homozygous mutation in the *ATM* gene, which codes for a serine/threonine kinase involved in DNA repair[77]. Monoallelic A*TM* mutation carrier status, an estimated 1.4% of the United States population, is also associated with an increased risk of cancer, especially that of the female breast[78,79]. Among the families of those with AT, the rate of PDAC is at least twice that of the general population[80,81]. A 2012 study of a familial pancreatic cancer cohort found monoallelic ATM mutations in 2.4% of the PDAC probands, and that number increased to 4.6% of the patients with at least 3 FDRs with PDAC. Loss of heterozygosity of the *ATM* gene was found in the only patient with available tumor tissue in the study[77].

Non-O blood group: Non-O blood groups have also been associated with a higher risk of PDAC[82-84]. Multiple prospective and case-control studies across different countries as well as a genome-wide association study demonstrated an increased risk of PDAC among those with non-O blood groups; additionally, a 2010 meta-analysis found that having an O blood group was associated with a relative risk of 0.79 for the development of PDAC[83,85]. In fact, it was demonstrated that each additional non-O allele conferred a larger risk of PDAC[86]. Interestingly, it was shown that the association between non-O blood groups and PDAC was largest in individuals colonized by CagA-negative *Helicobacter pylori* (*H. pylori*)[84]. While it has been postulated that the increased cancer risk is related to a chronic host inflammatory state, it has been found in one study that non-O blood groups do not increase the risk of chronic pancreatitis[83,87].

FPC: Unknown gene: Familial pancreatic cancer (FPC), defined as having 2 or more FDRs with PDAC with no known genetic cause, is responsible for up to roughly 80% of clustering PDAC[3]. The National Familial Pancreas Tumor Registry at Johns Hopkins demonstrated a nine-fold greater risk of developing PDAC among individuals with an FDR with PDAC in the setting of FPC, compared to a 1.8-fold greater risk for those with an FDR with sporadic PDAC[12]. Additionally, among FPC kindreds, having two or three FDRs with PDAC was associated with a 6.4-fold and 32-fold greater risk of developing PDAC, respectively.

Additionally, studies of the European Registry of Hereditary Pancreatitis and FPC as well as the German National Case Collection for FPC Registries have described anticipation (developing PDAC roughly 10 years earlier than their affected parent) in 59%-80% of over 100 FPC families[33,88]. Finally, segregation analyses have shown evidence for a yet-unidentified autosomal dominant, high-risk allele influencing the onset age of PDAC present in 7/1000 individuals[89]. The *palladin* gene, a proto-oncogene overexpressed in some sporadic pancreatic tumors has also been found to be mutated in affected members of one PDAC family[90-92]. This gene codes for a cytoskeleton protein that promotes tumor invasion in fibroblasts[90].

PDAC risk factors: Modifiable

Multiple modifiable risk factors are associated with an increased risk of developing PDAC (Table 2). Since PDAC has such a low incidence rate and most of the associated relative risks (with the exception of chronic pancreatitis) are low, greater improvements in PDAC morbidity and mortality may be possible with lifestyle modification.

Tobacco use: Smoking is the largest identifiable and modifiable risk factor for PDAC, contributing to 20%-35% of PDAC cases[93-95]. A 2008 meta-analysis of 82 studies demonstrated an increased risk of PDAC development for both current cigarette (relative risk of 1.74) and pipe or cigar (1.47) users[93]. A 2012 pooled analysis found the risk of current cigarette use to be 2.2-fold[96]. Additionally, both studies found increased smoking intensity and cumulative smoking dose to increase the risk for development of PDAC. Even after 10 years of smoking cessation, a modestly elevated relative risk of 1.48 remains[93]. However, multiple studies have demonstrated a risk of PDAC among former smokers to be similar to non-smokers after up to 15-20 years of cessation[96-100]. Finally, exposure to second-hand tobacco smoke has been found to increase the risk of PDAC by 21%[101].

It is likely that PDAC develops from exposure to tobacco-related carcinogens through circulating blood, especially given a similar rate of tobacco-related neoplasm in the kidney and stomach[93]. These carcinogens, including nitrosamines and polycyclic aromatic hydrocarbons, as well as their metabolites, cause mutations in both protoncogenes (K-ras) and tumor suppressors (p53)[102,103]. Tobacco smoke also directly contributes to pancreatic inflammation[103].

Smoking is particularly harmful in certain cohorts. For patients with HP, smoking has been demonstrated to more than double the risk of PDAC and lower the age of cancer onset by 20 years[95]. For members of FPC families, one study found cigarette smoking resulted in a 4-fold increased risk over non-smokers, as well as lowering the age of onset of PDAC by 10 years[104]. Another study demonstrated an incidence ratio of 19.2 for members of PDAC families who had ever smoked cigarettes *vs* 6.25 for those who had never smoked at all[12].

Alcohol use: While alcohol has been found to be associated with PDAC, the current evidence indicates that it is limited to heavy alcohol usage only: pooled data and meta-analyses have found three or more drinks per day to be associated with a 1.22 to 1.36-fold increased risk of developing PDAC, with a dose-response relationship[105,106]. It is known that heavy alcohol usage does contribute to pancreatitis, which may be a method by which it increases the risk of PDAC[107]. Additionally, metabolites of alcohol, including acetaldehyde (a carcinogen) and fatty acid ethyl esters, as well as ethanol itself (a carcinogen) can cause pancreatic inflammation as well as directly contribute to carcinogenesis[103].

Chronic pancreatitis: A 2010 meta-analysis demonstrated a relative risk of 13.3 for developing PDAC in those with chronic pancreatitis, with a ten to twenty year lag between the incidences of pancreatitis and pancreatic malignancy[69]. As with hereditary pancreatitis, chronic inflammation seen in chronic pancreatitis is thought to be the mechanism by which PDAC develops. Far and away, the most common cause of chronic pancreatitis is alcohol abuse, which is responsible for 60%-90% of cases[108]. As with HP, chronic inflammation is thought to be the mechanism by which PDAC develops in chronic pancreatitis. Inflammatory cytokines may induce cellular proliferation, as well as reduce immunosurveillance and inhibit

senescence, allowing the lesion to continue to grow[73].

Diet and obesity: Meta-analyses have demonstrated an increased risk of PDAC associated with a diet including red meat in men (relative risk of 1.29), and processed meat in both men and women (1.19)[109]. Another meta-analysis found that there was a relative risk of 1.12 for developing PDAC for each 5kg/m^2 increase in body mass index (BMI)[110]. A large 2003 study found a BMI of over 40 to be associated with a relative risk of PDAC of 1.49 for men and 2.76 for women[111]. Interestingly, a 2009 study found being overweight or obese at a younger age to be associated with a younger age of onset of PDAC; the study also found those who had a BMI over 25 from ages of 30 to 79 had reduced PDAC survival[112]. The method by which fat consumption may lead to PDAC includes pancreatic hypertrophy and hyperplasia in response to cholecystokinin-mediated lipase secretion from the presence of fat in the duodenum, which puts the pancreatic exocrine glands at an increased risk of carcinogenesis[102]. Additionally, hyperglycemia, abnormal glucose levels, and insulin resistance are all associated with an increased risk of PDAC[112-117].

Diabetes mellitus: type 1, type 2, type 3c: Meta-analyses have demonstrated associations between both type 1 and type 2 diabetes mellitus (DM) and pancreatic cancer, with odds ratios of approximately 2.0 and 1.8, respectively[109,118,119]. Twenty-five to 50% of patients with PDAC will have developed DM 1-3 years prior to their PDAC diagnosis; however, the relative risk of pancreatic cancer drops as time from type 2 DM diagnosis increases, indicating that DM may in fact be an early manifestation of the cancer[118,120,121]. Also, while new-onset DM is not specific for PDAC (less than 1% of adult-onset DM patients will develop PDAC within 3 years), large cohort studies in the United States and Sweden have demonstrated differing relative risks for those with a long history of DM *vs* those with new-onset DM: having DM for a longer time is associated with a decreased PDAC risk compared to newly-diagnosed DM[121-124]. In addition, associated new-onset DM has been shown to resolve after tumor resection[114,125,126].

A different diabetes diagnosis, type 3c (pancreatogenic) DM, or diabetes associated with acute or chronic disease of the pancreas, which is up to 8% of all diabetes, may confer an even higher risk of pancreatic cancer, especially in those patients with chronic pancreatitis[121,127-129]. Type 3c DM occurs in up to 30% of patients with PDAC and is associated with deficiencies in islet hormones such as insulin, glucagon, and pancreatic polypeptide[121]. Most frequently, the insulin resistance is actually hepatic resistance, with relatively normal peripheral insulin sensitivity; this is thought to be a result of a deficiency of pancreatic polypeptide, which has been shown to affect hepatic insulin receptors[128,130]. In patients with pancreatic polypeptide deficiency, this hepatic insulin resistance has been shown to return to normal with the replacement of the hormone[128,131,132].

Insulin is growth promoting, and thus chronic insulinemia may result in increased cellular proliferation and decreased apoptosis, a mechanism by which PDAC may eventually develop[110,112,117]. This is mediated through both increased levels of insulin, as well as insulin-like growth factor-1, which also results from hyper-insulinemia[102]. Additionally, the oxidative stress from hyperglycemia may be the cause of cell damage that could lead to the development of neoplasm.

DM treatment choice has been demonstrated to modulate pancreatic risk. One case-control study found a relative risk of 2.89 for pancreatic cancer in those with DM; this risk decreased to 2.12 with treatment by oral hypoglycemic agents and increased to 6.49 by treatment with insulin[98]. This is consistent with evidence that insulin can promote pancreatic cancer cell proliferation[133]. In particular, treatment with metformin has been shown to decrease overall cancer risk in diabetic patients[134,135]. Multiple studies have demonstrated a decreased risk of pancreatic cancer among diabetics treated with metformin[135-137]. Specifically, one study demonstrated that treatment with metformin conferred a relative risk of pancreatic cancer of 0.30, *vs* 2.78 with treatment with insulin[135].

Surgery and infection: A meta-analysis found a relative risk of PDAC of 1.23 for those with a history of a cholecystectomy[138]. The mechanisms suggested by which cholecystectomy increases the risk of PDAC include increased cholecystokinin levels, which have been shown to stimulate the growth of human pancreatic cancer cell lines and promote pancreatic carcinogenesis in hamsters, as well as increased degradation of bile salts to secondary bile acids, which have a pancreatic carcinogenic effect in hamsters[138-142].

Another meta-analysis has demonstrated a relative risk of 1.54 for developing PDAC post-gastrectomy, with a higher risk found for Billroth Ⅱ resections than Billroth Ⅰ resections[143,144]. The reasons postulated for higher rates of pancreatic carcinogenesis include a post-gastrectomy environment favorable for bacteria that increase levels of DNA-damaging N-nitrosamine carcinogens, increased rates of *H. pylori* seropositivity, and increased rates of recurrent acute pancreatitis in Billroth Ⅱ resections[144].

Evidence suggests *H. pylori* infection is associated with PDAC: a 2011 meta-analysis found an increased odds ratio of 1.38[145]. The definitive method by which *H. pylori* infection contributes to the development of PDAC is unknown, but may be related to the inflammatory mediators and angiogenic factor secretion associated with chronic infection[145]. There is some evidence for a link between hepatitis B infection and pancreatic cancer, as well as possibly hepatitis C; however, the method by which these infections contribute to PDAC is unknown[146,147].

Hydrocarbon exposure: While studies have shown correlations between pancreatic cancer and various expo-

sures, the most consistent exposures linked to development of pancreatic neoplasm are chlorinated hydrocarbons and polycyclic aromatic hydrocarbons[148]. However, it is important to note that consistently statistically significant results have not been found with either of these two occupational exposures.

PDAC STAGING, RISK STRATIFICATION AND SCREENING

Staging, prognosis, and the case for screening

The five-year PDAC survival rate of 6% is dismal, largely because the majority of patients are diagnosed at an advanced stage[1]. Surgical resection is the only curative treatment for pancreatic cancer. However, only pre-cancerous or early-stage (Ⅰ-Ⅱ) PDAC is surgically resectable. Since five-year survival rate for patients diagnosed with Stage Ⅰ A disease is 19 times that of those diagnosed with Stage Ⅳ disease (13.6% *vs* 0.7%), greater improvements in survival may be seen if we focus on shifting the diagnosis of PDAC from a late stage to an early or pre-cancerous stage[8]. Unfortunately, early-stage PDAC is usually clinically silent, highlighting the need for improved methods of early detection of precursor and early stage lesions. This provides the rationale for screening programs to detect precursor and early stage lesions.

PDAC precursors

World Health Organization guidelines suggest that in order to screen for a cancer, there must be a recognizable latent or early stage of the disease that can be tested for and managed effectively[148]. Several pancreatic lesions meet the criteria for a precursor to PDAC: pancreatic intraepithelial neoplasms (PanINs), mucinous cystic neoplasms (MCNs), and intraductal mucinous cystic neoplasm (IPMNs)[149,150].

PanIN: PanINs are non-invasive, non-mucin-producing, small epithelial neoplasms[150,151]. There are 3 grades of PanINs, classified by degree of atypia: PanIN-1, PanIN-2, and PanIN-3. A 2003 study found PanIN lesions in 82% of pancreata with invasive cancer compared to just 28% of normal pancreata, as well as an increased number of high-grade PanIN lesions compared to low-grade PanIN lesions[152]. Multiple studies have found PanIN-3 lesions only in pancreata harboring other malignancies[152-154]. For PanIN lesions, there are three broad subsets of germline or somatic mutations that are usually found in concert in a pancreatic malignancy: (1) activation of oncogenes (*K-Ras, HER2*); (2) inactivation of tumor suppressor genes (*TP53*, *p16/CDKN2A*, *SMAD4/DPC4, BRCA1, BRCA2*); and (3) inactivation of genome maintenance genes (*MLH1*, *MSH2*)[151,155,156]. While PanINs are not visible on cross-sectional imaging, a 2006 study suggests that endoscopic ultrasound (EUS) may be able to detect lobular parenchymal atrophy associated with PanINs, particularly multifocal PanIN, and IPMNs[157].

Pancreatic cystic neoplasms: MCN and IPMN: Autopsies indicate that the prevalence of patients with a pancreatic lesion at death is about 24%; studies have found that magnetic resonance imaging (MRI) picks up incidental pancreatic cysts in patients with no pancreatic history in up to 13.5% of patients, and computed tomography (CT) in 2.6%[158-160]. The ability to detect precursor lesions before they invade and progress to pancreatic cancer is of the utmost importance. MCNs are cystic, mucin-producing epithelial neoplasms with ovarian-type stroma, detectable on cross-sectional imaging[150]. MCNs are much more common in females than males (95% female), and a significant percentage of the stroma cells stain positive for estrogen or progesterone receptors[161,162]. With a mean age of diagnosis of 45-50, MCNs usually arise in the body or tail of the pancreas (> 90%) and do not communicate with the larger pancreatic ducts[161-165]. Compared to non-invasive MCNs, malignant MCNs are diagnosed in older patients and are significantly larger, indicating that they most likely grow slowly over time[163,166]. The five-year survival rate for margin-negative, surgically resected non-invasive MCNs is close to 100%, but roughly 50% for invasive MCNs; however, their low frequency of invasion (12%) highlights the need for better characterization of tumor progression[161-163,166].

IPMNs, which include branch duct (BD-IPMN), main duct (MD-IPMN), and mixed types, are mucin-producing epithelial neoplasms that are also detected by cross-sectional imaging[167]. They are more common in the head of the pancreas, affect men more than women and have a mean age of diagnosis of about 65 years of age[166,168]. While BD-IPMNs and MD-IPMNs have the same age of presentation, BD-IPMNs are more common and frequently multifocal (21%-41% of cases) and less likely to progress to malignancy (11%-17% *vs* 44%-48% *vs* 45% for mixed IPMNs)[166,169-173]. Patients with resected BD-IPMNs also have a higher five-year survival rate (91%) than both MD-IPMNs (65%) and mixed IPMNs (77%)[166].

Patients with both MCNs and IPMNs have improved survival when lesions are resected before developing an invasive component: a study of 851 consecutive resected patients at Massachusetts General Hospital showed a five-year survival rate of 87% for those with invasive and non-invasive cystic lesions and just 62% in those with malignancy[172].

While it is important to continue to better our ability to identify these PDAC precursor lesions, this must be matched by an improvement in the capacity to accurately predict which of those lesions will progress to malignancies. Characterizing how these precursor lesions develop will help better guide future screening and subsequent treatment.

Screening modalities: Imaging and biomarkers

Imaging: EUS and MRI have demonstrated the most accuracy as screening modalities for PDAC in terms of detecting small, cystic lesions, while magnetic resonance

Table 3 Pancreatic ductal adenocarcinomas screening efforts and diagnostic yields *n* (%)

Ref.	Number screened	High-risk group	Initial imaging (if abnormal screening)	Diagnostic yield	Definition of diagnostic yield
Brentnall *et al*[182]	14	FPC	EUS + ERCP + CT	7 (50)	Dysplasia
Rulyak *et al*[183]	35	FPC	If symptomatic: EUS + ERCP If asymptomatic: EUS (ERCP)	12 (34.3)	Dysplasia
Kimmey *et al*[184]	46	FPC	EUS (ERCP)	12 (26)	Dysplasia
Canto *et al*[185]	38	FPC, PJS	EUS (CT, ERCP, EUS-FNA)	2 (5.3)	PDAC, IPMN
Canto *et al*[186]	78	FPC, PJS	EUS + CT (ERCP, EUS-FNA)	8 (10.3)	IPMN, PanIN1-2
Poley *et al*[187]	44	FPC, BRCA, PJS, FAMMM, p53, HP	EUS (CT, MRI)	10 (23)	PDAC, IPMN on imaging
Langer *et al*[188]	76	FPC, BRCA, FAMMM	EUS + MRCP (EUS)	1 (1.3)	IPMN
Verna *et al*[181]	51	FPC, PJS, FAMMM, BRCA, HP, HNPCC	EUS and/or MRCP (EUS-FNA, ERCP)	6 (12)[1]	PDAC, IPMN, multifocal PanIN2-3
Ludwig *et al*[189]	109	FPC, BRCA	MRCP (EUS)	9 (8.3)	PDAC, IPMN, PanIN2-3, SCA on imaging
Vasen *et al*[190]	79	p16	MRI/MRCP, EUS if unable	7 (8.9)	PDAC
Al-Sukhni *et al*[191]	262	FPC, FDR of double-primary cancer, BRCA, PJS, HP, p16	MRI (ERCP, EUS, CT)	3 (1.1)[2]	PDAC
Schneider *et al*[33]	72	FPC, BRCA, PALB2, p16	EUS + MRCP (EUS)	4 (5.5)	MD-IMPN, multifocal PanIN23
				9 (12.5)	MD-IMPN, multifocal PanIN2-3, BD-IPMN
Canto *et al*[174]	216	FPC, BRCA, PJS	CT + MRI/MRCP + EUS (ERCP)	92 (42.6)	Pancreatic lesion

[1]Only 41 patients had imaging, resulting in yield of 14.6% (6/41); [2]Only 175 patients had imaging, resulting in yield of 1.7% (3/175). PDAC: Pancreatic ductal adenocarcinomas; HNPCC: Lynch syndrome; FAP: Familial adenomatous polyposis; PJS: Peutz-Jeghers syndrome; FAMMM: Familial atypical multiple mole melanoma syndrome; HP: Hereditary pancreatitis; FPC: Familial pancreatic cancer; endoscopic retrograde MRI: Magnetic resonance imaging; CT: Computed tomography; EUS: Endoscopic ultrasonography; ERCP: Endoscopic retrograde cholangiopancreatography; MCN: Mucinous cystic neoplasms ; IPMN: Intraductal mucinous cystic neoplasm; FNA: Fine needle aspirate.

cholangiopancreatography (MRCP) provides the best visualization of possible communication with the main pancreatic duct[9,174]. CT subjects patients to radiation and has a suboptimal detection rate compared to EUS and MRI. Abdominal ultrasound and endoscopic retrograde cholangiopancreatography are not used as screening modalities for PDAC[9].

Biomarkers: Due to high cost, relative inability of non-invasive imaging modalities to detect small and solid tumors, and the modest risks associated with screening techniques like EUS, the use of biomarkers for the early detection of PDAC is an important frontier[175].

Carbohydrate antigen 19-9 (CA 19-9) is the only FDA approved blood biomarker test for PDAC[176]. However, due to the low prevalence of PDAC in the population, CA 19-9 is recognized as a poor screening tool: a screening of over 10000 patients found only 4 cases of PDAC based on CA 19-9 levels; additionally, 3 of those cases were not resectable at diagnosis[176]. The sensitivity (70%), specificity (87%), positive predictive value (59%), and negative predictive value (92%) are still not high enough to be used regularly in healthy patients[176,177]. CA 19-9 levels do appear to be informative as a predictor of disease recurrence post-resection[176,178].

The literature surrounding pancreatic cancer biomarkers is vast: a 2009 analysis found over 2500 genes overexpressed at the mRNA or protein level[179]. There is ongoing research that suggests a future for gene expression profiling, proteomics, metabolomics, and microRNA as diagnostic PDAC biomarkers.

Current screening guidelines

The low absolute risk of developing PDAC precludes population-wide screening at the current time, both from a cost-benefit and absolute harm perspective. Assuming a lifetime risk of developing PDAC of 1.49%, a hypothetical screening test with 90% sensitivity and specificity would have a positive predictive value (PPV) of just 12%, meaning that almost nine in ten positive screening results would be incorrect, with those patients subject to unnecessary stress and further testing[3]. Even a screening test with 95% sensitivity and specificity would result in a PPV of just 22%. Notwithstanding, the identification of genetic and environmental risk factors may provide opportunities to enrich the screening population with high-risk cohorts, which would drastically increase the PPV of screening results, with the hopes of identifying precursor or early-stage lesions in some high-risk individuals before the lesions progress to inoperable pancreatic cancer.

Brand *et al*[180] published recommendations for PDAC screening in 2007. They suggested that potential candidates for screening included: (1) *BRCA1, BRCA2*, *p16* mutation carriers with at least one FDR or SDR with PDAC; (2) a PJS family member (preferably confirmed germline mutation carrier); (3) HP patients; (4) a patient with 2 relatives in same lineage with PDAC, at least one of whom is an FDR of the patient; and (5) patients with $\geqslant$ 3 FDR, SDR or third-degree relatives with PDAC. They suggested that screening of these individuals

Table 4 Selected highlights

Selected recent advances	Genetic risk factors
	In 2009, the use of gene sequencing identified PALB2, which had previously been implicated in breast cancer, as a susceptibility gene for PDAC[28]
	Expression of the palladin gene has been shown to be upregulated by cohabitance of normal fibroblasts with epithelial cells expressing the K-Ras oncogene. In 2012, it was shown that the palladin gene, which codes for a cytoskeletal protein, promotes mechanisms for metastasis and outgrowth of tumerogenic cells[90]
	Also in 2012, gene sequencing indicated that ATM mutations result in a predisposition to PDAC; LOH was demonstrated in 2 kindreds with PDAC[77]
	Therapy
	For patients with diabetes, treatment with metformin is associated with a lower relative risk of pancreatic cancer[127,136,137]
	A 2011 case report detailing a complete pathological response of a BRCA2-associated pancreatic tumor to gemcitabine plus iniparib showed the potential for PARP inhibitors in the treatment of BRCA2-associated pancreatic cancer[41]. Similar clinical trials are currently underway
Screening	Screening goals
	The goal of PDAC screening is the detection and treatment of (1) resectable PDAC; (2) PanIN-3 lesions; and (3) IPMN with high-grade dysplasia
	Low prevalence and high risk cohort enrichment
	The low absolute risk of PDAC development precludes population-wide screening from a cost-benefit and absolute harm perspective. The opportunity to screen high-risk cohorts will vastly increase the PPV of a screening test
	Screening efforts
	Past screening efforts, using patients cohorts at a high risk of developing PDAC, have demonstrated diagnostic yields from 1.1% to 50%, depending on their definition of yield (Table 3). Current screening modalities may be costly and invasive, and therefore associated with some patient risk. Furthermore, the long-term implications for detection of small and clinically insignificant lesions are uncertain. Further studies are needed to determine appropriate surveillance
Anticipated future advances and screening possibilities	Risk stratification
	Personal, family, genetic and environmental history will allow risk stratification and development of tailored screening and surveillance programs
	Biomarkers
	Ongoing research that suggests a future for gene expression profiling, proteomics, metabolomics, and microRNA as diagnostic PDAC biomarkers
	Targeted therapy
	As with BRCA2-associated tumors and PARP inhibitors, tumor biology will increasingly dictate the subsequent therapy

PDAC: Pancreatic ductal adenocarcinomas; IPMN: Intraductal mucinous cystic neoplasm.

should occur only under research protocol conditions, and required a threshold of at least 10-fold increased risk of PDAC. However, there was no consensus on the approach to screening, when to begin screening, and frequency of surveillance.

In 2011, the International Cancer of the Pancreas Screening (CAPS) Consortium held a conference with a panel of 49 experts from multiple disciplines, with the goal "to develop consortium statements on screening, surveillance and management of high-risk individuals with an inherited predisposition to PC [pancreatic cancer]"[9]. There was agreement that detecting and treating invasive resectable PDAC as well as multifocal PanIN-3 and IPMN with high-grade dysplasia should be considered a successful outcome of a screening or surveillance program.

The CAPS consortium suggested guidelines for PDAC screening, based on evidence of increased PDAC risk[9]. The statements agreed upon (> 75% consensus) were to screen candidates with: (1) two FDRs with PDAC; (2) two blood relatives with PDAC and at least one FDR; (3) PJS; (4) *BRCA2* mutation carriers with either one FDR with PDAC or at least two affected family members; (5) *PALB2* mutation carriers with at least one FDR with PDAC; (6) *p16* mutation carriers (FAMMM) with at least one FDR with PDAC; and (7) lynch syndrome and one FDR with PDAC. While they agreed that initial screening should include EUS and/or MRI/MRCP, there was no consensus about when to start or end screening.

Risk stratification

Based on personal and family history and genetic testing, patients can be stratified into risk categories. Verna *et al*[181] defined average risk patients as having one family member with PDAC, diagnosed at age 55 or older; these patients do not receive screening with EUS or MRI. Moderate risk patients were defined as those with two or more first, second, or third-degree relatives with PDAC, or an FDR with PDAC diagnosed earlier than age 55; these patients are screened with EUS or MRI. Finally, high risk patients had three or more first, second, or third-degree relatives with PDAC, two or more FDRs with PDAC, one FDR and one SDR with PDAC one of whom was diagnosed before age 55, or a genetic syndrome with PDAC associated with it; these patients receive both EUS and MRI. For all of the risk groups, any abnormal testing is followed by EUS if not already done. Following this screening, if no malignant or premalignant

disease is found, the patient is surveilled based on their risk factors. If malignant or premalignant disease is suspected or diagnosed, surgery must be considered.

Past PDAC screening efforts

A number of PDAC screening programs directed at various high-risk groups have been published, largely focusing on EUS as a screening modality. While each group screened individuals only at elevated risk of PDAC, inclusion criteria, screening modalities, and definition of diagnostic yield varied across groups, resulting in a wide range of reported yields. Their results, with diagnostic yields ranging from 1.1% to 50%, can be found in Table 3[3,9].

CONCLUSION

PDAC is the fourth most common cause of cancer-related deaths in the United States and a major health issue[1]. With dismal five-year survival rates, significant advances in the understanding of the etiology and tumor biology, as well as early detection, screening and treatment of PDAC are needed (Table 4). Given that only those diagnosed at an early or precancerous stage have a reasonable expectation of low morbidity and mortality, increased efforts are needed to improve risk stratification and identify early stage disease or premalignant conditions while they are still resectable. PDAC screening efforts in these enriched cohorts may also allow us to identify more effective modalities for early detection and screening, which could be then modified and instituted in the general population.

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P- Reviewer: Westgaard A **S- Editor**: Zhai HH
L- Editor: A **E- Editor**: Wang CH

World Journal of Gastroenterology

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DOI: 10.3748/wjg.v20.i32.11199

World J Gastroenterol 2014 August 28; 20(32): 11199-11209
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (14): Pancreatic cancer

MicroRNAs as emerging biomarkers and therapeutic targets for pancreatic cancer

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Received: October 24, 2013 Revised: March 6, 2014
Accepted: June 13, 2014
Published online: August 28, 2014

Abstract

Despite tremendous efforts from scientists and clinicians worldwide, pancreatic adenocarcinoma (PDAC) remains a deadly disease due to the lack of early diagnostic tools and reliable therapeutic approaches. Consequently, a majority of patients (80%) display an advanced disease that results in a low resection rate leading to an overall median survival of less than 6 months. Accordingly, robust markers for the early diagnosis and prognosis of pancreatic cancer, or markers indicative of survival and/or metastatic disease are desperately needed to help alleviate the dismal prognosis of this cancer. In addition, the discovery of new therapeutic targets is mandatory to design effective treatments. In this review, we will highlight the translational studies demonstrating that microRNAs may soon translate into clinical applications as long-awaited screening tools and therapeutic targets for PDAC.

Key words: MicroRNAs; Biomarkers; Pancreatic cancer; Therapeutic targets; Precancerous lesions

Core tip: Robust biomarkers and reliable treatments are needed to help alleviate the dismal prognosis of pancreatic cancer. In this review, we will highlight the translational studies demonstrating that microRNAs may soon translate into clinical applications as long-awaited screening tools and therapeutic targets for this cancer.

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PANCREATIC CANCER

There are currently no means for the reliable diagnosis of early stages of pancreatic cancer (PDAC) and the curative treatment of late stages. Consequently, the vast majority

of patients (80%) display an advanced disease that results in a low resection rate leading to a dismal overall median survival of less than 6 mo[1]. The estimated 5-year survival rate is lower than 2%. While PDAC is not among the most common tumors, it is one of the most frequent causes of cancer-related death with approximately 40000 death/year in the United States and in Europe. Thus, there is an urgent need to discover diagnostic as well as prognostic molecular markers together with reliable therapeutics to improve pancreatic cancer management.

PDAC is a highly heterogeneous disease[2] defined by numerous alterations in multiple signaling pathways[3]. Additionally, specific cellular clones for primary tumors and metastasis have been identified[4]. Interestingly, the type and number of genomic rearrangements in DNA vary considerably between patients, and occur early during tumor development[5]. On the other end, pioneering studies using genome-wide profiling showed that microRNAs (miRNA) expression can discriminate cancers with high efficacy[6]. In this review, we will focus on the use of miRNAs as promising biomarkers and therapeutic targets for pancreatic cancer (Tables 1-3).

GENERAL CONCEPT OF MIRNAS AND CANCER

miRNAs are small RNA molecules that functions as translation inhibitors of messenger RNA by their binding to 3'-untranslated region[7-9]. These molecules are tightly involved in the regulation of many physiological processes such as development, proliferation, invasion, and apoptosis among others. Interestingly, their expression is profoundly altered in cancer and/or is strongly modulated during carcinogenesis. Thus, the activation of tumor-suppressive miRNAs and the inhibition of oncogenic miRNAs by small molecules or gene transfer may have the potential to provide a fundamentally new approach for the development of cancer therapeutics. Probably the most important advantage in comparison with current approaches targeting single genes is the ability to modulate many different pathways "at once" taking into account that one miRNA can regulate hundreds of genes, frequently in the context of a cell-specific network.

MIRNAS AS DIAGNOSTIC MARKERS FOR PANCREATIC CANCER

To date, many strategies based on high-throughput screening are used to discover relevant clinical biomarkers. For PDAC in particular and pancreatic tissue in general, these protocols are often hindered by the intrinsic high levels of many nucleases. Consequently, the high stability of miRNAs in tissues and fluids is a key advantage over protein and mRNA. In addition, miRNAs can be quantified in very low amounts of material and in highly degraded samples, such as small biopsies and fine needle aspirates. This is mandatory to support the use of miRNAs as biomarkers for PDAC at the clinical level. In the next sections, we will update the excellent reviews[2,10-15] and meta-analysis[16] from other groups, and reviews and book chapters we recently published[17-19] on the use of miRNAs as biomarkers in PDAC (Tables 1 and 2).

Historically, Pr Schmittgen's group was the first to report the expression profiles of miRNAs in PDAC. They identified miRNAs specifically over expressed in PDAC (miR-376a, miR-301) or in other tumors (miR-155, miR-21, miR-221 and miR-222)[20]. Two additional miRNAs (miR-132 and miR-212) were recently reported to be over expressed in PDAC as compared to normal or benign adjacent pancreas to the tumor[21]. Another study by Pr Shao's group yielded conflicting results as they demonstrated that miR-132 was down regulated in cancer *vs* normal benign normal tissues[22]. Other miRNAs, such as miR-96[23], miR-34a[24] and miR-21[25], have been reported to be altered in PDAC as compared to normal adjacent tissue. miRNAs expression may also help to discriminate PDAC from chronic pancreatitis. This is of particular importance to prevent from unnecessary and possibly debilitating surgery, or to delay tumor treatment, respectively. Historically, Pr Bloomston's group reported that 21 miRNAs with increased expression and 4 underexpressed miRNAs differentiated PDAC from normal tissue in 90% of samples and from pancreatitis with 93% accuracy[26]. Twenty additional miRNAs were discovered by Szafranska *et al*[27] to discriminate between PDAC, chronic pancreatic and normal pancreas. Later, expression of miR-203[28], miR-148a[29], miR-196b[29], miR-196a[29] and miR-205[29] were demonstrated to be altered in PDAC *vs* chronic pancreatitis. Alternatively, miRNA expression profiles have been recently used to distinguish PDAC from cholangiocarcinoma, two virtually indistinguishable cancers using conventional histopathological and clinical characteristics[30].

Endoscopic ultrasound-guided fine needle aspirations (FNA) material allows for the screening of the vast majority (> 85%) of PDAC patients that are not eligible for surgery, and, as consequence, may provide new insights for the diagnosis and prognosis of PDAC. Pr Szafranska's group was the first to demonstrate that the expression of miR-196a and miR-217 in FNA material can classify PDAC from benign lesion[31]. This pioneering study led to the development of the first molecular test for the identification of PDAC[32]. Hence, we demonstrated that *let-7* miRNA expression is repressed in PDAC FNAs[33], and that the measurement of hypermethylation of miR-148a encoding DNA region is potentially useful to differentiate PDAC and pseudo-tumor forms of chronic pancreatitis[34].

MIRNAS AS PROGNOSTIC AND PREDICTIVE MARKERS FOR PANCREATIC CANCER

MiRNAs are also scrutinized for their ability to predict cancer prognosis and/or response to treatment. Bloomston *et al*[26] were the first to report that miR-452,

Table 1 MicroRNAs as diagnostic markers for pancreatic cancer

miRNA	Biopsies	FNA	Circulating	Ref.
Let-7a	X (↓)	X (↓)		[33]
miR-34a	X (↓)			[24]
miR-96	X (↓)			[23]
miR-99a			X (↑)	[55]
miR-101	X (↑)			[67]
miR-132	X (↑/↓)			[21,22]
miR-141	X (↓)			[27]
miR-143	X (↑)			[27]
miR-145	X (↑)			[27]
miR-146a	X (↑)			[27]
miR-148a	X (↓)			[27,29]
miR-148b	X (↓)	X (↓)		[27,34]
miR-150	X (↑)			[27]
miR155	X (↑)		X (↑)	[26,27,53,62,67,68]
miR-16			X (↑)	[57]
miR-181a	X (↑)			[26]
miR-181b	X (↑)			[26]
miR-181d	X (↑)			[26]
miR-185			X (↑)	[59]
miR-191			X (↑)	[55,59]
miR-196a	X (↑)	X (↑)	X (↑)	[27,29,32,53,57]
miR-196b	X (↑)			[27,29]
miR-20a			X (↑)	[55,59]
miR-200a			X (↑)	[56]
miR-200b			X (↑)	[56]
miR-203	X (↑)			[28]
miR-210	X (↑)		X (↑)	[29,54]
miR-212	X (↑)			[20]
miR-216	X (↓)			[27]
miR-217	X (↓)	X (↓)		[27,31]
miR-21	X (↑)	X (↑)	X (↑)	[20,26,25,51,53,55,60,64, 67-69]
miR-210	X (↑)		X (↑)	[27,53]
miR-221	X (↑)		X (↑)	[20,26,58,69]
miR-222	X (↑)			[20,26,27]
miR-223	X (↑)			[27]
miR-24			X (↑)	[55]
miR-27a-3p			X (↑)	[59]
miR-29c	X (↓)			[27]
miR-30a-3p	X (↓)			[27]
miR-301	X (↑)			[20]
miR-31	X (↑)			[27]
miR-375	X (↓)			[27]
miR-376a	X (↑)			[20]
miR-494	X (↓)			[27]
miR-1290	X (↑)			[61]

miRNAs: MicroRNAs; Biopsies: Resected tumors; FNA: Fine needle aspiration; ↑: Upregulated; ↓: Downregulated.

miR-105, miR-127, miR-518a-2, miR-187, and miR-30a-3p are over-expressed in the tumors of patients with survival greater than 2 years. Moreover, tumors with high expression of miR-196a-2 or miR-219 have a lower median survival compared with those with low expression. In addition, over expression of miR-155[35], miR-200[35], miR-203[35], miR-205[35], miR-200c[36], miR-21[37], miR-212[38] and miR-675[38] and reduced expression of miR-34a[37], miR-30d[37], miR-148a[38], miR-187[38], miR-130b[39] and *let-7g*[38] in PDAC are associated with poorer survival rate. Last, low miR-211 expression was demonstrated as an independent factor of poor prognosis in resected PDAC[40].

Gemcitabine is broadly used as a first-line chemotherapeutic treatment for patients with unresectable locally advanced or metastatic pancreatic cancer[41]. However, the 5-year survival rate is only 2%[42], with 1-year survival rates ranging from 17% to 23%[41]. Recently, phase Ⅱ and Ⅲ trials exploring gemcitabine-based combinations with erlotinib[43], FOLFIRINOX[44] or nab-Paclitaxel[45] were found to improve overall survival of patients. However, the moderate activity of standard gemcitabine and gemcitabine-based regimens still encourages the discovery of robust biomarkers that may help to stratify PDAC patients for tailored therapy. Gemcitabine requires transporter proteins to cross cell membranes. Low expression of human equilibrative nucleoside transporter-1 (hENT1) may result in gemcitabine resistance in PDAC. Recent studies have revealed that high levels of hENT1 in PDAC predict longer survival times in patients treated with adjuvant gemcitabine[46]. In another study, CO-101, a lipid-drug conjugate of gemcitabine, was designed to enter cells independently of hENT1[47]. However, CO-101 was found not superior to gemcitabine in patients with metastatic PDAC and low tumor hENT1. In addition, metastasis hENT1 expression doesn't predict gemcitabine outcome. Interestingly, Giovannetti *et al*[48] found that high miR-21 expression in tumors is associated with shorter overall survival both in the metastatic and in the adjuvant setting, while patients with low miR-21 expression may benefit from gemcitabine treatment[49]. Gemcitabine resistance is also associated with the cellular over expression of miR-146 and the reduced expression of miR-205 and miR-7[50]. Last but not least, Pr Korc's group recently demonstrated that miR-10b is a novel and powerful diagnostic biomarker for PDAC[51]. Like miR-21, miR-10b is over expressed in the FNA material from PDAC patients. Additionally, reduced expression of miR-10b is associated with improved response to multimodality neoadjuvant therapy, likelihood of surgical resection, delayed time to metastasis, and increased survival. Thus, miR-10b is likely to be a novel marker to diagnose PDAC, but may also serve as a biomarker for response to gemcitabine-based neoadjuvant therapy, and be predictive of early metastasis formation. In experimental models, miR-10b was demonstrated to promote PDAC-derived cells proliferation and invasion by suppressing TIP30, which enhances EGFR signaling, facilitates EGF-TGF-β cross-talk together with the expression of epithelial-mesenchymal transition-promoting genes[52].

CIRCULATING MIRNAS AS BIOMARKERS FOR PANCREATIC CANCER

The recent discovery of miRNAs in serum or plasma opens up the possibility of using non coding RNAs as circulating biomarkers of disease. Wang *et al*[53] were the first to report the detection of miRNA in the blood of PDAC patients. They demonstrated that plasmatic miR-21, miR-210, miR-196a and miR-155 reveal a sensi-

Table 2 MicroRNAs as prognostic and predictive markers for pancreatic cancer

miRNA	Biopsies	FNA	Prognostic	Predictive of treatment efficacy	Ref.
miR-105, miR-127, miR-187, miR-30a-3p, miR-452, miR-518a-2	X (↑)		+		[26]
miR-155, miR-200, miR-203, miR-205	X (↑)		+		[35]
miR-21 (↑), miR-34a (↓), miR-30d (↓)	X		-		[37]
miR-212 (↑), miR-675 (↑), miR-148a (↓), miR-187 (↓), let-7g (↓)	X		-		[38]
miR-146 (↑), miR-205 (↓), miR-7 (↓)	X		-	-	[50]
miR-10b		X (↑)		-	[51,52]
miR-196a	X (↑)		-		[26]
miR-219	X (↑)		-		[36]
miR-200c	X (↑)		+		[36]
miR-21	X (↑)	X (↑)	-	-	[40,41,51]

miRNAs: MicroRNAs; Biopsies: Resected tumors; FNA: Fine needle aspiration; ↑: Upregulated; ↓: Downregulated; +: Good prognosis/response to treatment; -: Bad prognosis/response to treatment.

Table 3 MicroRNAs as therapeutic targets in pancreatic cancer

miRNA	Expression	Known target(s)	Function	Ref.
Let-7	↓	KRAS	Inhibition of cell proliferation, chemosensitization	[33,85,86]
miR-10b	↑	TIP30	Increased cell proliferation and invasion	[52]
miR-21	↑		Inhibition of cell proliferation, invasion, tumor growth, chemoresistance and inhibition of apoptosis	[95]
miR-23b			Radioresistance	[91]
miR-29a		Wnt/β-catenin	Chemosensitization to gemcitabine	[88]
miR-34a	↓	Smad3	Inhibition of cell proliferation and invasion, induction of apoptosis	[82,83,94]
miR-96	↓	KRAS	Inhibition of cell proliferation, invasion, tumor growth and induction of apoptosis	[23,70]
miR-99b		mTOR	Radioresistance	[90]
miR-132	↑↓	Rb1	Alteration of cell proliferation	[31,32]
miR-138		lipocalin	Inhibition of tumorigenicity	[93]
miR-141	↓	MAP4K4	Inhibition of cell proliferation and invasion, chemosensitization	[92]
miR-142-3p			Inhibition of cell proliferation	[72]
miR-148a	↓		None	[78]
miR-148b	↓	AMPKα1	Inhibition of cell proliferation, invasion and chemosensitization	[79]
miR-150*		IGF-1R	Induction of apoptosis	[73]
miR-181b	↑	NFkB	Chemosensitization to gemcitabine	[89]
miR-197			Induction of EMT	[75]
miR-198	↓	MSLN, OCT-2, PBX-1, VCP	Inhibition of cell proliferation, invasion, tumor growth and induction of apoptosis	[71]
miR-212	↑	Rb1	Increased cell proliferation	[21]
miR-218	↓		Inhibition of cell proliferation and tumor growth and metastasis	[84]
miR-221	↑		Increased migration, proliferation and EMT	[77]
miR-320c		SMARCC1	Chemosensitization to gemcitabine	[77]
miR-373			Increased tumor growth	[74]
miR-630		IGF-1R	Induction of apoptosis	[73]
miR-655			Inhibition of EMT	[76]

↑: Upregulated; ↓: Downregulated; EMT: Epithelial-mesenchymal transition.

tivity of 64% and a specificity of 89% for PDAC. A recent study further confirmed that circulating miR-210[54] and miR-21[55] are elevated in PDAC patients and may potentially serve as a useful biomarker for PDAC diagnosis. In addition, miR-200a[56], miR-200b[56], miR-16[57], miR-196a[57], miR-20a[55], miR-24[55], miR-25[55], miR-99a[55], miR-185[55], miR-221[58] and miR-191[55] were described as significantly elevated in the sera of PDAC as compared with controls. Combining miR-16[57] and miR-196a[57], or miR-27a-3p[59] detection with CA 19-9 quantification is even more effective to discriminate PDAC from controls. However, Pr Hoheisel's group recently reported that blood miRNAs profile could not discriminate pancreatitis from PDAC efficiently[60]. Last, but not least, Pr Goggin's group recently demonstrated that miR-1290 accurately distinguishes patients with low-stage pancreatic cancer from healthy and disease controls[61]. Such study paves the way for the non-invasive detection of early PDAC lesions.

OPEN QUESTION: WHAT IS THE SIGNIFICIANCE OF MIRNAS IN HIGH RISK PATIENTS FOR DEVELOPING PANCREATIC CANCER?

One of the current avenues of research to improve the management of pancreatic cancer is to better understand the early stages of the disease in order to allow for curative surgery and to prevent the risk of cancer in popula-

tions at risk. Advances in biomedical research have led to recent evidence that pancreatic cancer develops from preneoplastic lesions which can be considered as very effective risk factors. Three types of lesions have been identified so far: pancreatic intraepithelial neoplasia, mucinous cystadenomas, and Intraductal Papillary Mucinous Neoplasia of the pancreas (IPMN). Interestingly, the latter lesions can be readily detected due to the progress and the multiplicity of the imaging devices in the clinical departments. The risk of degeneration of IPMNs varies according to the type of injured duct: it is of the order of 60% for IPMN located in the main duct (or mixed) while this risk is estimated at 15% for branch ducts. IPMN now represent 25% of the diagnosed pancreatic cystic tumors and 20% of resected pancreatic tumors, respectively. Therefore, one of the most promising strategies to improve the dismal prognosis of pancreatic cancer is to identify early indicators of degeneration of IPMNs in populations at high risk of developing this cancer. Interestingly, miRNAs have recently revealed a great potential as reliable early diagnosis biomarkers in IPMNs. Again, miR-21 and miR-155 are highly expressed in IPMN, while miR-155 is elevated in IPMN-associated pancreatic juice as compared to controls[62]. We demonstrated that miR-205 and miR-21 overexpression precede phenotypic changes in the pancreatic ducts, both in human samples and in transgenic mice developing cancer[63]. Interestingly, such over expression may occur early in the transformation from normal pancreatic tissue, as benign cystic tumors of low and high malignant potential express high levels of this miRNA[64]. This strongly suggests that miRNAs such as miR-21 can possibly be used for an early diagnosis of this neoplasm. In a similar experimental model, Yabushita *et al*[65] recently reported the over expression of miR-155, miR-21, miR-210, miR-18a, miR-203, miR-30b-5p, miR-31, miR369-5p, miR3-376a and miR-541 in the serum of a human KRAS oncogenic transgenic rat model. More importantly, Matthaei *et al*[66] assessed the diagnostic benefit of using miRNAs as biomarkers in pancreatic cyst fluid in patients, to identify IPMN that require resection and exclude non-mucinous cysts with a sensitivity of 89%, a specificity of 100%, and AUC of 1. This work was further completed by Pr Giovannetti's group who demonstrated that miR-21, miR-155 and miR-101 showed significant differences in invasive *vs* non-invasive IPMNs, with miR-21 described as an independent prognostic biomarker in invasive IPMNs[67]. Again, miR-21 and miR-155 were recently described as upregulated during the development and progression of IPMN[68]. MiR-21 in cystic fluid was identified as a candidate biomarker to distinguish between benign, premalignant, and malignant cysts[69], while miR-221 could be used for the identification of more advanced malignant disease[69]. Last, a work from Pr Maitra's group recently revealed that a 9-miRNA panel quantified in cystic fluid may aid in diagnosis and surgical treatment decisions for patients with pancreatic cystic lesions, such as high-grade IPMNs[66]. Thus, miRNAs may reveal as non-invasive indicators of degeneration in a population at high risk of developing incurable cancer. Once identified, patients will be stratified and will benefit from early surgical management that will greatly improve their survival and prognosis. Finally, this approach is likely to strengthen the surveillance protocol and to reduce the costs associated with patients care.

ROLE OF MIRNAS IN PANCREATIC CANCER

miRNAs are broadly involved in pancreatic carcinogenesis

Many miRNAs have been reported to alter cancer proliferation and/or migration, both *in vitro* and *in vivo*. miR-132 and miR-212 were recently reported to be over expressed in pancreatic cancer as compared to normal or benign adjacent pancreas to the tumor[21]. Interestingly, these miRNAs target the retinoblastoma tumor suppressor 1 (Rb1) to favor cancer cell proliferation[21]. Another study by Pr Shao's group yielded conflicting results as they demonstrated that miR-132 was down regulated in cancer *vs* normal benign normal tissues[22]. In the later study, enforced expression of miR-132 in cell lines derived from PDAC led to proliferation and colony formation inhibition[22]. Yu *et al*[23] reported that miR-96 is downregulated in PDAC as compared to normal tissues and targets KRAS. Consequently, restoring miR-96 expression strongly inhibited *in vitro* cell proliferation, invasion, induced apoptosis and reduced tumor growth. This was further confirmed in a recent study linking ecotropic viral integration site 1 oncoprotein-mediated inhibition of miR-96 to promote KRAS expression during early pancreatic carcinogenesis[70]. MiR-198 acts as a central tumor suppressor in PDAC and modulates the expression of many oncogenic factors such as MSLN, OCT-2, PBX-1, and VCP[71]. Very interestingly, low miR-198 expression prognosticates poor patient outcome, while high miR-198 may disrupt this oncogenic network and predict better prognosis and increased survival.

Epigenetic regulation of miRNAs involved in pancreatic cancer progression

MiR-148 family members may have distinct effects on PDAC-derived cells proliferation. While miR-148a expression is lost during PDAC carcinogenesis following methylation of its DNA sequence[34], we recently demonstrated that enforced expression of this miRNA didn't impaired PDAC-derived cells cell proliferation nor tumor growth in experimental models[72]. On the other hand, recent results described that miR-148b can inhibit cell proliferation, invasion, and enhance chemosensitivity of PDAC by targeting AMPKα1[73]. MiR-124 is also silenced by aberrant methylation in PDAC; consequently, tumor progression and metastasis are enhanced due to the lack of Rac1 targeting[74]. MiR-34a miRNA, which is directly regulated by p53, is also subjected to epigenetic silencing in numerous neoplasms, including PDAC[75]. Strikingly, this miRNA plays a pivotal role in PDAC stem cell self-

renewal and may hold significant promise as novel target for PDAC[24]. In addition, the natural compound genistein up-regulates this miRNA to suppress cell proliferation and induce cell death by apoptosis of PDAC-derived cell lines[76]. MiR-34a was also recently reported as a tumor metastasis suppressor by negatively modulating Smad3[77]. Last, Li *et al*[78] recently demonstrated that the histone methyltransferase enhancer of zeste homolog 2 inhibits miR-218 expression, that prevents proliferation of PDAC cells in culture, and tumor growth and metastasis in nude mice.

miRNAs regulates the epithelial-mesenchymal transition in pancreatic cancer

Besides the miR-200 family members (reviewed elsewhere), miRNAs such as miR-197 and miR-655 have been recently involved in the epithelial-mesenchymal transition in PDAC cells, by targeting p120 catenin[79] and ZEB1 and TGFBR2, respectively[80]. In addition, microRNA-221 participates in the effects of PDGF-BB on migration, proliferation, and to the epithelial-mesenchymal transition in these cells[81].

miRNAs are key players in drug-mediated inhibition of pancreatic cancer growth

Recently, different molecules were found to alter miRNA expression in PDAC to inhibit cell proliferation and/or tumor growth. Triptolide that downregulates HSP70, a molecular chaperone upregulated in several tumor types, was recently shown to upregulate miR-142-3p in PDAC cells, to inhibit cell proliferation[82]. More importantly, Minnelide, a water-soluble prodrug of triptolide, induces the expression of miR-142-3p *in vivo*. In addition, the adamantyl retinoid-related molecule 3-Cl-AHPC was recently demonstrated to induce miR-150* and miR-630 miRNAs expression to target IGF-1R and promote apoptosis in PDAC cells[83]. Inappropriate regulation of intracellular zinc levels may also plays an important role in PDAC. Recently, increased zinc influx mediated by the zinc importer ZIP4 was demonstrated to induce miR-373 expression in pancreatic cancer to promote tumor growth[84].

Besides miR-148b, *Let-7* is also involved in the chemosensitization of PDAC-derived cell lines. Indeed, reduced expression of the *let-7* miRNAs family members was identified in gemcitabine-resistant PDAC cell lines[85]. This was correlated with a higher expression of ribonucleotide reductase subunit M2 (RRM2), a key protein involved in gemcitabine resistance. In this work, the authors nicely demonstrated that *Let-7* can regulate RRM2 expression, but also that *Let-7* biogenesis was severely impaired in PDAC cells[85]. The latter effect seems to be recurrent in PDAC as nuclear TRAILR2 was recently demonstrated to inhibit maturation of *Let-7* in PDAC cell lines to increase their proliferation[86]. Additionally, miR-320c, miR-29a and miR-181b were found to regulate the resistance of PDAC cells to gemcitabine through SMARCC1[87], the Wnt/β-catenin[88] and the NF-κB[89] signaling pathways, respectively. In a recent report, miR-141 was found to target MAP4K4 to inhibit cell proliferation, clonogenicity and invasion, induce G1 arrest and apoptosis, and enhance chemosensitivity[90]. Alternatively, radiation resistance of PDAC-derived cell lines has also been linked to miRNAs, such as miR-99b[91]. In a very interesting study by Wang *et al*[92], miR-23b was found to regulate autophagy associated with radioresistance of PDAC cells.

MIRNAS AS NEW THERAPEUTIC TARGETS FOR PANCREATIC CANCER MANAGEMENT

As stated in the previous sections, miRNA expression is profoundly altered in pancreatic cancer and/or is strongly modulated during carcinogenesis. Thus, the activation of tumor-suppressive miRNAs and the inhibition of oncogenic miRNAs may have the potential to provide a fundamentally new approach for the development of therapeutics for many cancers including PDAC. Probably the most important advantage in comparison with current approaches targeting single genes is the ability to modulate many different pathways "at once" taking into account that one miRNA can regulate hundreds of genes, frequently in the context of a cell-specific network. In this section, we will update our recent book chapters on the use of miRNAs as therapeutic tools to control PDAC progression[17,18] (Table 3).

Few reports described the use of miRNAs as therapeutic targets to control PDAC tumor progression, *in vivo*. We demonstrated that *let-7* enforced expression strongly inhibits PDAC cell proliferation[33]. This was achieved either using plasmid-encoding miRNA or lentiviral vectors. However, restoring *let-7* levels in cancer-derived cell lines failed to impede tumor growth progression after intratumoral gene transfer. Using a similar strategy, Lee *et al*[93] recently demonstrated that miR-138 transfection of cancer cells *in vivo* reduces tumor formation by targeting neutrophil gelatinase-associated lipocalin. Interestingly, nanoparticles targeted to PDAC-derived cells using bifunctional CC9 peptide successfully delivered miR-34a to inhibit the growth of subcutaneous PANC-1 tumors[94]. We recently devised a lentiviral vector to target miR-21, one of the most described miRNA in oncology[95]. Following transduction with this vector, PDAC-derived cells cell proliferation is strongly inhibited, and cancer cells die by apoptosis through the mitochondrial pathway. *In vivo*, a single inoculation of the therapeutic vectors in exponentially growing PDAC tumors stops cancer progression, inhibits cell proliferation and provokes cancer cell death by apoptosis. We found that our approach surpasses the therapeutic efficacy of standard treatments for this disease. Interestingly, miR-21 depletion enhances tumor angiogenesis; consequently, combining miR-21 targeting with gemcitabine eradicate experimental PDAC tumors. During this study, we treated existing tumors with miR-21

antagonists, a paradigm closely related to the clinical scenarios in which such therapies will be employed. While there clearly remains significant work to be done, this work is the first to demonstrate that targeting oncogenic miRNA is very effective to stop the tumor growth of a very aggressive PDAC model. It also emphasizes the central role of miR-21 in this cancer, and paves the way to forthcoming studies to discover the many pathways controlled by this miRNA in PDAC. Because miR-21 is over expressed in most human tumors; therapeutic delivery of miR-21 antagonists may still be beneficial for a large number of cancers for which no cure is available.

CONCLUSION

miRNAs can be detected and quantified not only in frozen tissues, but also in formalin-fixed paraffin-embedded tissues, as well as serum and plasma samples. These tiny but potent molecular markers have proven effective for PDAC classification, prognostic stratification and drug-response prediction. Strikingly, miR-21, and to a lower extent miR-196, miR-217, miR-10b and miR-155, appears to be constantly up regulated in PDAC, and to be indicative of poor survival, response to treatment and/or metastatic disease. PDAC is also frequently associated with a dense stromal reaction that may favor tumor progression and resistance to treatment. Recently, Pr Donahue's group has pointed out that miR-21 expression in PDAC tumor-associated fibroblasts is associated with decreased overall survival and promotes tumor cells invasion[96]. This work may stem for novel diagnostic and therapeutic strategies for dual targeting of both tumor and stroma in PDAC. Whether this will translate into clinical applications is still highly debated. Above all, circulating miRNAs, in combination with other "omics" approaches such as proteomics, are expected in the future to prove specific and/or sensitive as a long-awaited screening tool for PDAC.

On the other hand, miRNAs are key players in PDAC carcinogenesis, and can be organized in oncogenic networks aimed at inhibiting multiple tumor suppressor genes. They are involved in the regulation in many if not all cancerous pathways such as cell proliferation, dissemination, resistance to apoptosis or chemotherapy. Consequently, the development of miRNA-based therapies have the potential to overcome the limitations of present cancer therapies that often lead to relapse because of the complexity and the redundancy of the targeted signaling pathways. The path from drug discovery to clinical trials is long and still hampered by many challenges. Despite the fact that hundreds of ongoing clinical trials include miRNA as biomarkers, miR-122 is the unique miRNA that as successfully reached clinical trial as targeted therapy to treat HCV infection[97,98]. Nevertheless, it is our belief that miRNA-based therapeutics (especially to target miR-21) for cancer are not far behind, and that combination of miRNA therapy with targeted or traditional therapies may provoke a synergistic effect for treatment of cancer in clinical trials in the next few years.

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P- Reviewer: Andersson RG, Fernandez-Zapico ME, Hu R, Lee KT, Yip-Schneider MT **S- Editor**: Ma YJ **L- Editor**: A **E- Editor**: Zhang DN

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DOI: 10.3748/wjg.v20.i32.11210

World J Gastroenterol 2014 August 28; 20(32): 11210-11215
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (14): Pancreatic cancer

Selection criteria in resectable pancreatic cancer: A biological and morphological approach

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Received: November 5, 2013 Revised: February 13, 2014
Accepted: April 15, 2014
Published online: August 28, 2014

Abstract

Pancreatic ductal adenocarcinoma (PDA) remains one of the most aggressive tumors with a low rate of survival. Surgery is the only curative treatment for PDA, although only 20% of patients are resectable at diagnosis. During the last decade there was an improvement in survival in patients affected by PDA, possibly explained by the advances in cancer therapy and by improve patient selection by pancreatic surgeons. It is necessary to select patients not only on the basis of surgical resectability, but also on the basis of the biological nature of the tumor. Specific preoperative criteria can be identified in order to select patients who will benefit from surgical resection. Duration of symptoms and level of carbohydrate antigen 19.9 in resectable disease should be considered to avoid R1 resection and early relapse. Radiological assessment can help surgeons to distinguish resectable disease from borderline resectable disease and locally advanced pancreatic cancer. Better patient selection can increase survival rate and neoadjuvant treatment can help surgeons select patients who will benefit from surgery.

Key words: Pancreatic ductal adenocarcinoma; Pancreatic cancer; Borderline resectable pancreatic cancer; Pancreatic surgery; Pancreatic cancer staging

Core tip: The aim of this work was to improve identification of patients with pancreatic ductal adenocarcinoma, who will benefit from pancreatic resection. Duration of symptoms and level of carbohydrate antigen 19.9 in patients with resectable disease should be considered to avoid R1 resection and early relapse. Radiological assessment can help surgeons to distinguish resectable disease from borderline resectable disease and locally advanced pancreatic cancer.

Tamburrino D, Partelli S, Crippa S, Manzoni A, Maurizi A, Falconi M. Selection criteria in resectable pancreatic cancer: A biological and morphological approach. *World J Gastroenterol* 2014; 20(32): 11210-11215 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11210.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11210

INTRODUCTION

Despite recent advances in cancer therapy, pancreatic ductal adenocarcinoma (PDA) remains one of the most aggressive tumors and is among the four most frequent causes of tumor-associated deaths in both men and women in the European Union and the United States[1,2]. Surgical resection still represents the only curative treatment for PDA, although only a small fraction of tumors is amenable to surgical resection at diagnosis[3-6]. Moreover, among patients who undergo surgery, 30% develop early recurrence as a result of misdiagnosed aggressive disease[6]. The aim of this paper is to review the current available data on factors related to adverse prognosis in

patients with resectable PDA.

EPIDEMIOLOGY

Only 20% of patients with PDA are resectable at diagnosis and 5-year overall survival (OS) after curative resection is only 20%[4-8]. During the last decade survival rates of PDA have remained dismal with a 5-year OS of 15%-20% after pancreaticoduodenectomy and 8%-15% after distal pancreatectomy[9,10]. In the 1990s there was no improvement in 5-year OS, which was even lower (2.3%-2.7%) compared with the 5-year OS rate observed in the late 1980s (2.5%-3.1%)[11]. Despite progress in diagnostic procedures, most cases are still metastatic at diagnosis, and are not amenable to radical surgery and even when curative surgery is performed, most patients will eventually relapse[11]. In a large, retrospective, study of a high-volume centre in Italy, Barugola *et al*[12] compared the survival time-trends in a selected population of patients affected by resectable PDA. There were 114 (21%) resections in 1990-1999 and 430 (79%) in 2000-2008. The length of hospital of stay (16 d *vs* 10 d) and postoperative mortality (2.6% *vs* 1.1%) significantly decreased over time. The median disease-specific survival significantly increased from 16 mo in the first period to 29 mo in the second period. Resection performed in 1990-2000 was an independent predictor of poor outcome, indicating that long-term survival after surgery for resectable PDA significantly improved in the last decade. This improvement is possibly explained by the advances in cancer therapy but also by better patient selection by pancreatic surgeons. As regards oncological progress, in recent years several efforts have been made to develop effective drugs for pancreatic cancer. In particular, two recent randomized clinical trials that included patients with metastatic PDA demonstrated significantly better survival for the treatment groups compared with control groups of patients treated with gemcitabine[13,14]. Conroy *et al*[14] showed that patients treated with FOLFIRINOX (5-fluorouracil, oxaliplatin, and irinotecan) had improved survival compared with a gemcitabine alone group, with a median OS of 11.1 mo *vs* 6.8 mo with an objective response rate of 31.6% *vs* 9.4%. Similarly, Von Hoff *et al*[13] have shown a better survival in patients with PDA treated with gemcitabine plus nab-paclitaxel compared with gemcitabine alone. In this work, OS was 8.5 mo in the treatment group compared with 6.7 mo in the control group. The increase in objective response rate due to improvement in oncological treatments can also have the consequence of increasing the number of resectable patients[15]. Better patient selection has probably modified the survival of patients with PDA because of changing resectability criteria. Among those who undergo surgical resection, up to 30% of patients die of disease within 1 year after surgery[6,16]. In this subgroup, recurrence is early, and survival rates are comparable to those observed in patients with advanced disease undergoing antitumoral therapies alone[17]. The risk of early failure after surgery could be associated with the following: (1) inadequate preoperative radiological staging; (2) lack of radical surgery; and (3) differences in tumor aggressiveness. Undoubtedly, what is common to patients who will recur early, is disease with more aggressive biological behavior.

All of these patients are resectable at diagnosis, but probably the difference with the others patients is the biological characteristics of the tumor. In addition, there is a relationship between hospital volume with long-term survival in patients with cancer subjected to pancreatectomy, probably due to patient selection and technical expertise at the major centers that are responsible[18]. Therefore, it is necessary to select patients not only on the basis of surgical resectability, but also on the basis of the biological nature of the tumor.

Preoperatively, we can identify specific criteria to be recognized in order to select those patients who will actually benefit from surgical resection. Focusing on these criteria, we suggest a step-by-step approach for patients with pancreatic cancer; the first step is to consider their clinical and laboratory factors and then their radiological features.

CLINICAL AND LABORATORY CRITERIA

In order to select patients who will benefit from a surgical approach, we have to consider not only the imaging but also other parameters such as symptoms, risk of mortality related to the patient's comorbidity, and the level of carbohydrate antigen (CA),19.9. Symptoms of PDA depend on the site of the pancreatic lesion; for pancreatic head tumors, jaundice is the first sign, whereas for pancreatic body/tail tumors, pain is the most frequent symptom. Duration of symptoms > 40 d is an important parameter associated with a higher risk of early recurrence among patients who undergo surgery[6]. Although the reason behind abdominal pain in PDA remains unclear, it is likely that this represents the result of pancreatitis or tumoral invasion of the retroperitoneal nerves[10,19,20]. The presence of invasion of the retroperitoneal nerves, which causes pain, means that the tumor is over the gland, thus, despite radiological resectability, it should be considered as a borderline or locally advance disease. Nevertheless, not all patients with a resectable PDA are also fit for surgery. Before planning pancreatic resection therefore, it is mandatory to assess carefully the surgical risk of each patient. Several studies have demonstrated that elderly patients have an increased risk of morbidity after pancreaticoduodenectomy (PD), in particular related to postoperative pancreatic fistula, although morbidity and mortality rates are acceptable[21]. It could be therefore justified to offer PD to elderly patients who do not have significant comorbidity[21]. Brozzetti *et al*[22] have compared two group of patients (Group A > 70 years and Group B < 70 years). They showed significantly higher operative morbidity and mortality in Group A and they concluded that, although an aggressive surgical approach is justified

in elderly patients with pancreatic adenocarcinoma, surgical complications that lead to reoperation are responsible for high mortality in elderly patients. In addition to general causes, such as concomitant disorders, reduced functional reserve, poor tolerance to stress, and the texture of the pancreatic remnant, there are specific prognostic factors affecting pancreaticojejunostomy leakage and related mortality.

Another important parameter related to the aggressiveness of disease is the level of CA19.9. CA19.9 has been used for the diagnosis, prognosis, and follow-up of pancreatic cancer patients. Preoperative CA19-9 is strongly associated with tumor stage. A decrease in CA19.9 level is the best index of improved prognosis[23,24]. In contrast, patients with increased CA19.9 after resection had a significantly shorter median survival time. In another study published by Montgomery *et al*[25], patients who had CA19.9 < 180 U/mL in the first 3 mo after surgery had improved survival. Lower preoperative CA19.9 values correlated not only with a lower pathological stage, but also with increased post-resection survival. The presence of preoperative CA19.9 < 1000 U/mL was associated with a median survival of 28 mo compared with 12 mo in patients with CA19.9 > 1000 U/mL[23]. CA19.9 > 200 U/mL in patients with resectable PDA is associated with a higher risk of early failure after resection for pancreatic cancer. The importance of CA19.9 levels as a prognostic marker in PDA has been demonstrated in several other studies that have evaluated the decrease in CA 19-9 after anti-tumor therapy. Yang *et al*[26] have shown that patients who had a CA19.9 decrease of > 90% following chemoradiotherapy (CRT), had a significantly improved median survival compared with those who had not (16.2 mo *vs* 7.5 mo). The median survival of patients with a CA19.9 level lower than the median post-CRT value was 10.3 mo, compared with 7.1 mo for those with a CA19.9 level greater than the median. After CRT, CA19.9 < 50 U/mL also had a meaningful prognostic significance. In the neoadjuvant therapy setting, the measurement of CA19.9 is an essential variable in the evaluation of possible surgical resection of tumors that exhibit a response to treatment.

RADIOLOGICAL CRITERIA

The diagnostic phase and the resectability assessment of PDA should always involve a multidisciplinary evaluation. In this setting, it is important to offer patients the expertise of a high-volume center and dedicated multidisciplinary team (MDT). The importance of MDTs has been widely demonstrated for other malignancies[27,28]. Similarly, Pawlik *et al*[29] have analyzed the impact of MDTs in the management of patients with pancreatic cancer. They analyzed 203 patients with computed tomography (CT) that revealed locally advanced/unresectable disease (35%), metastatic disease (18%), and locally advanced disease with metastasis (1%). After an accurate review of the imaging, the clinical stage of the disease was modified in 19% of patients. Overall, 48 out of 203 (24%) patients had a change in their recommended management based on clinical review of their case by the pancreatic MDT. As a consequence, the quality of imaging as well as the expertise of radiologists contributes significantly to better patient selection. Imaging should include at least one high-quality technique such as CT or magnetic resonance imaging. CT should be performed according to a defined pancreas protocol such as triphasic cross-sectional imaging and thin slices. Optimal multiphase imaging techniques include a non-contrast phase, plus arterial, pancreatic parenchymal and portal venous phases of contrast enhancement with thin cuts (3 mm) through the abdomen[30]. The arterial phase shows excellent opacification of the celiac axis and the superior mesenteric artery, whereas the superior mesenteric, portal and splenic veins and the pancreas itself are opacified in the venous phase. Likewise, the detection of liver metastasis is optimal in the latter phase. Weg *et al*[31] and Kopka and Grabbe[32] have noted that a slice thickness of 2-4 mm is superior to 5-10 mm in the detection of small liver metastases. Moreover, the introduction of multidetector CT imaging has allowed the acquisition of these thinner slices in liver imaging, resulting in improved detection rates of liver metastases[33]. Vascular involvement is another important finding that can be assessed preoperatively by CT scan. A classification of vascular involvement in pancreatic cancer has been defined by the MD Anderson Group[34]. This classification includes two separate entities: (1) borderline resectable: PDA that is defined as a tumor with an abutment ⩽ 180° (one half or less) of the circumference of the superior mesenteric artery (SMA) and/or with a short-segment encasement/abutment of the common hepatic artery (typically at the gastroduodenal origin) and/or with short-segment occlusion with suitable vessel above and below in superior mesenteric vein (SMV) or portal vein (PV); and (2) locally advanced: PDA that is defined as a tumor with an encasement > 180° of the SMA and/or with an encasement and no technical option for reconstruction usually because of extension to the celiac axis/splenic/left gastric junction or the celiac origin, and/or with occlusion of the SMV/PV without an option for reconstruction. Nonoperative management for locally advanced pancreatic cancer (LAPC) is largely accepted[15,35-37]. Neoadjuvant treatment with combination chemotherapy results in a higher resection rate compared with single agent chemotherapy (33% *vs* 27%) as confirmed by Gillen *et al*[38] in their meta-analysis. In contrast, the optimal management for borderline resectable tumors is still debated. Compared with resectable PDA, borderline tumor is characterized by a higher risk of positive-margin resection with a subsequent higher risk of recurrence[34]. Although the prognosis of borderline resectable patients is significantly better than that of LAPC, survival rates are worse than those of resectable tumors[39]. Moreover, the role of arterial resection (AR) during pancreatectomy in borderline tumors has been analyzed in a recent systematic review published by

Mollberg *et al*[40]. Perioperative morbidity rates of patients with AR ranged from 17% to 100% (median 53.6%) with a median mortality rate of 12% (range: 0%-45.5%) compared to 2.6% in standard pancreatic resection[29,30]. Pancreatectomy with AR then increases the risk of mortality fivefold, without significant advantages in terms of long-term survival. These results demonstrate that the artery involvement by PDA, implies a more aggressive tumor biology, and these neoplasms should be considered as locally advanced despite the feasibility of surgical resection. Also, the involvement of the splenic artery has been demonstrated to be an adverse prognostic factor in body/tail PDA[41]. Neoadjuvant therapy is specifically beneficial in borderline resectable tumors and increases the fraction of resectable tumors. Katz *et al*[42] reported that 78% of patients completed neoadjuvant therapy and restaging, and 41% of them eventually underwent pancreatectomy. In this light, they suggest that neoadjuvant treatment could be considered to select properly patients who can benefit from surgery.

FURTHER DIAGNOSTIC TOOLS TO ASSESS RESECTABILITY

In several cases of patients with seemingly resectable tumors, clinical and radiological work-up could be lacking and further examinations are warranted in order to clarify doubtful findings (*i.e.*, elevated CA19.9 or persistence of abdominal pain). It has been observed that, in about 15% of patients with radiologically resectable PDA, surgery does not improve survival[43]. These patients are at high risk of early death despite radical surgery and they should be identified preoperatively using additional tests. Endoscopic ultrasound (EUS) is complementary to CT in the staging of the disease and in the detection of vascular invasion (SMA, SMV, and celiac axis) and lymph node metastasis[44,45]. Also EUS with fine needle aspiration (FNA) is preferable to CT-guided FNA in patients with resectable disease because of better diagnostic yield, safety, and potentially lower risk of peritoneal seeding[30]. EUS could be also helpful for obtaining a cytological grading of the tumor preoperatively. Among patients with borderline resectable PDA, the presence of a poorly differentiated or anaplastic tumor is another factor that shifts the management toward neoadjuvant treatment[6]. Nevertheless, the accuracy of FNA in the assessment of tumor grading has not been validated so far. Diagnostic staging laparoscopy to rule out metastasis not visible at standard imaging is routinely used in some institutions prior to surgery or chemoradiation or in patients with high risk for disseminated disease. Selective use of laparoscopy may be more appropriate and will probably be a more cost-effective approach[46]. The role of positron emission tomography (PET) with ^{18}fluorodeoxyglucose is still unclear, although it may be considered after formal pancreatic CT protocol in patients with high risk of metastasis, but it is not a substitute for high-quality, contrast-enhanced CT[30]. Nowadays, PET-CT favorably alters management more often when used for therapeutic monitoring compared to staging or restaging[47].

Beyond these imaging techniques, genetic status of a pancreatic carcinoma can be used to predict widespread metastatic failure. Several studies have demonstrated that there are different genomic alterations in PDA[48,49]. The most important are point mutations of *KRAS, CDKN2A/p16*, *TP53*, amd *SMAD4/DPC4*. Yonezawa *et al*[50] have analyzed the genetic abnormalities in precursor lesions such as pancreatic intraepithelial neoplasia, intraductal papillary mucinous neoplasms, mucinous cystic neoplasms and their relation to PDA. They have found that KRAS mutation in PDA is 75%-100%, and SMAD4/DPC4 inactivation is seen in 55% of PDA patients. The low expression levels of SMAD4 are associated with an high rate of lymph node metastasis and poor survival[49]. Tanaka *et al*[51] have reported that loss of SMAD4 protein expression and chromosome 18q deletion were distinctly associated with metastasis. Determinations of DPC4 status at initial diagnosis may be of value in stratifying patients into treatment regimens related to local control *vs* systemic therapy[52]. Locally advanced carcinomas from patients with no documented metastatic disease uncommonly showed loss of DPC4 expression (22%) as compared with carcinomas from patients with extensive metastatic burden in which the rates of DPC4 loss approached 75%. In this setting, patients with DPC4-positive carcinomas would receive greater clinical benefit from intensive local control by CRT compared to patients with DPC4-negative carcinomas in which systemic chemotherapy alone may be more appropriate[53]. The advantage of SMAD4/DPC4 expression as a prognostic indicator is that it is potentially assessable preoperatively or during staging laparoscopy, whereas other factors, such as margins, perineural invasion and lymph node status are determined only after resection.

CONCLUSION

Surgical resection is still the only curative treatment for PDA. Oncological treatments have improved survival in patients with pancreatic cancer, also by increasing the rate of down staging and consequently of resectability. This improvement is probably also due to better patient selection by pancreatic surgeons. Nevertheless, current definitions of resectable, borderline resectable and locally advanced tumors are based only on radiological parameters and do not take into consideration the biology of the disease. Indeed, in borderline resectable disease a clear advantage in terms of survival has not been demonstrated for up-front surgery. Furthermore, surgery for borderline resectable is burdened by a high rate of morbidity and mortality that does not improve survival. In this light, a new concept of borderline pancreatic cancer has to include clinical and biological aspects (type and duration of symptoms, CA19.9 level, and immunohistochemistry). The selection of patients who will benefit from surgery has to be improved in the setting of an MDT discussion

that also considers further examinations.

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P- Reviewer: Chang BW, Hoskovec D, Katuchova J
S- Editor: Zhai HH **L- Editor**: Kerr C **E- Editor**: Wang CH

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DOI: 10.3748/wjg.v20.i32.11216

World J Gastroenterol 2014 August 28; 20(32): 11216-11229
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (14): Pancreatic cancer

Pancreatic cancer and its stroma: A conspiracy theory

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Supported by National Health and Medical Research Council of Australia, the Cancer Council of New South Wales and the Cancer Institute NSW
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Received: November 4, 2013 Revised: February 18, 2014
Accepted: April 15, 2014
Published online: August 28, 2014

Abstract

Pancreatic cancer is characterised by a prominent desmoplastic/stromal reaction that has received little attention until recent times. Given that treatments focusing on pancreatic cancer cells alone have failed to significantly improve patient outcome over many decades, research efforts have now moved to understanding the pathophysiology of the stromal reaction and its role in cancer progression. In this regard, our Group was the first to identify the cells (pancreatic stellate cells, PSCs) that produced the collagenous stroma of pancreatic cancer and to demonstrate that these cells interacted closely with cancer cells to facilitate local tumour growth and distant metastasis. Evidence is accumulating to indicate that stromal PSCs may also mediate angiogenesis, immune evasion and the well known resistance of pancreatic cancer to chemotherapy and radiotherapy. This review will summarise current knowledge regarding the critical role of pancreatic stellate cells and the stroma in pancreatic cancer biology and the therapeutic approaches being developed to target the stroma in a bid to improve the outcome of this devastating disease.

Key words: Pancreatic cancer; Stromal reaction; Tumour-stroma interactions; Pancreatic stellate cells; Metastasis

Core tip: This review summarises current knowledge about the role of pancreatic stellate cells in production of cancer stroma, the mechanisms mediating stromal-tumour interactions and novel therapeutic approaches developed on the basis of our increasing understanding of the critical influence of stromal elements on disease progression.

Xu Z, Pothula SP, Wilson JS, Apte MV. Pancreatic cancer and its stroma: A conspiracy theory. *World J Gastroenterol* 2014; 20(32): 11216-11229 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11216.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11216

INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer related death in developed countries[1,2]. From 1999 to 2008, the incidence rate of pancreatic cancer increased nearly 1% in United States, although the reason for this increase is unknown[3]. In general, pancreatic cancer refers to pancreatic ductal adenocarcinoma (PDAC) that accounts for around 90% of pancreatic cancer[4]. Since the clinical symptoms can be vague, patients are often diagnosed late, with regional invasion or distant metastasis already evident at first consultation[5-7]. The overall five-year survival rate of pancreatic cancer is approximately 6% in the United States[2], less than 6% across Europe[8] and 5% in Australia[9]. Despite the concerted endeavours

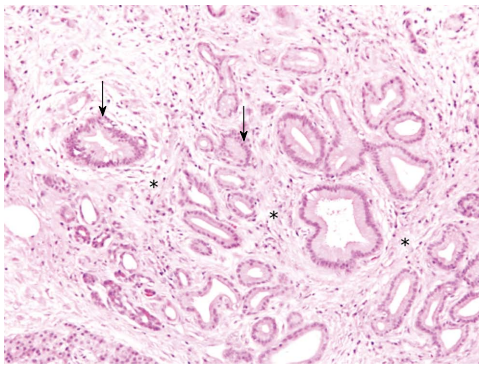

Figure 1 Pancreatic cancer and stromal reaction. A representative HE stained human pancreatic cancer tissue section showing duct-like and tubular structures (malignant elements, examples highlighted by arrows) infiltrating into and embedded in a highly fibrotic stromal reaction (examples highlighted by asterisks).

of clinicians and scientists over several decades, pancreatic cancer remains a devastating disease with a poor outcome.

Risk factors for pancreatic cancer include age, smoking, race, diabetes and chronic pancreatitis, the last being the strongest known risk factor for this disease. Patients with a more than 5 year history of chronic pancreatitis have a greater than 14-fold risk of developing pancreatic cancer compared to the general population[10,11]. 40% of hereditary pancreatitis (a form of chronic pancreatitis) patients are likely to develop pancreatic cancer[12-14], while patients with tropical pancreatitis have been reported to have a 100-fold increased risk and an earlier onset of the disease compared to sporadic cases[15,16]. The mechanisms underlying the increased propensity for patients with chronic pancreatitis to develop pancreatic cancer are not fully elucidated. Recent studies suggest several signalling pathways known to be active in inflammatory disease, may be involved in the progression from pancreatitis to pancreatic cancer.

One such signalling molecule known to play a key role in inflammation is the transcription factor, nuclear factor κB (NF-κB). Activation of NF-κB leads to the release of several proinflammatory cytokines, such as interleukin (IL)-1β, IL-6, tumour necrosis factor-alpha (TNF-α), transforming growth factor-beta (TGF-β) and induces anti-apoptotic responses *via* Bcl-xL[17]. In addition to its observed activation in pancreatitis, NF-κB activity has also been observed in pancreatic cancer tissue. It has been shown to modulate angiogenesis *via* vascular endothelial growth factor (VEGF) and urokinase, and apoptosis possibly *via* antiapoptotic proteins such as Bcl-xL, cIAP1 (inhibitor of apoptosis protein), cIAP2, TRAF1 (TNF receptor-associated factor) and TRAF2[10,18]. NF-κB also negatively regulates the expression of p53, which is a tumour suppressor gene[19]. Further evidence for a role of NF-κB in cancer comes from an *in vivo* study using a NF-κB inhibitor (LC-1) in a xenograft pancreatic cancer mouse model. This inhibitor was found to reduce tumour growth and was associated with decreased expression of cyclin D1, a protein required in cell cycle G1/S transition[18,20].

K-Ras is another signalling pathway that is involved in both chronic pancreatitis and pancreatic cancer. *K-Ras* mutations exist in about a third of chronic pancreatitis patients[21]. Daniluk *et al*[22] reported that oncogenic K-Ras activation by inflammation in the mouse pancreas promoted development of chronic pancreatitis and pancreatic cancer precursor lesions. In another study, mutant K-Ras in acinar cells resulted in neoplastic lesions in mouse pancreas that progressed to pancreatic cancer in conjunction with *p53* deletion[23]. Logsdon *et al*[24] have postulated that Ras activity is the direct link between chronic pancreatitis and pancreatic cancer. The induction of chronic pancreatitis in a genetically engineered mouse model with K-Ras overexpression led to the development of primary pancreatic tumours as well as metastasis[25-28]. Collins *et al*[29] have shown in mice bearing inducible *K-Ras* mutations, that oncogenic K-Ras initiates pancreatic carcinogenesis by hindering pancreatic repair after caerulein-induced pancreatitis. Importantly, inactivation of *K-Ras* mutation in these mice leads to tumour regression suggesting a role for oncogenic K-Ras in the maintenance of pancreatic cancer.

In addition to *K-Ras* mutations, a number of genetic mutations are frequently reported in pancreatic cancer. Biankin *et al*[30] performed exome sequencing and copy number analysis in a cohort of 142 sporadic PDAC cases and reported multiple significantly mutated genes, including the known mutations - *KRAS*, *TP53*, *CDKN2A*, *SMAD4*, *MLL3*, *TGFBR2*, *ARID1A*, *SF3B1* and importantly, previously unidentified mutations such as *EPC1*, *ARID2* (chromatin modifications), *ATM* (DNA damage repair), *ZIM2* (transcription regulation), *MAP2K4* (Toll-like receptor signalling pathway), *NALCN* (sodium channel activity), *SLC16A4* (monocarboxylate transporter), *MAGEA6* (protein binding). The accumulation of genetic mutations leads to the development of precursor lesions, the most common of which are pancreatic intraepithelial neoplasia (PanIN)[10,31,32]. PanINs are normally found in smaller diameter pancreatic ducts, with the microscopic features progressing from PanIN-1A to PanIN-3 and finally to overt PDAC.

Histopathologically, PDAC is characterised by duct-like and tubular structures (malignant elements) infiltrating into and embedded in a highly fibrotic stromal reaction[5,33] (Figure 1). This stromal reaction is comprised of abundant extracellular matrix (ECM), stromal cells, blood vessels/endothelial cells, immune cells, nerves/neurons and other soluble proteins, *e.g.*, cytokines, growth factors[10].

The ECM itself is composed of proteins such as type I collagen, fibronectin and laminin as well as proteoglycans, such as hyaluronan, which is a non-sulphated glycosaminoglycan secreted by cancer cells. Hyaluronan is known to bind to CD44 (its receptor) and to influence angiogenesis, epithelial-mesenchymal transition (EMT) and chemo-resistance, possibly *via* the regulation of receptor tyrosine kinase and small GTPase[34].

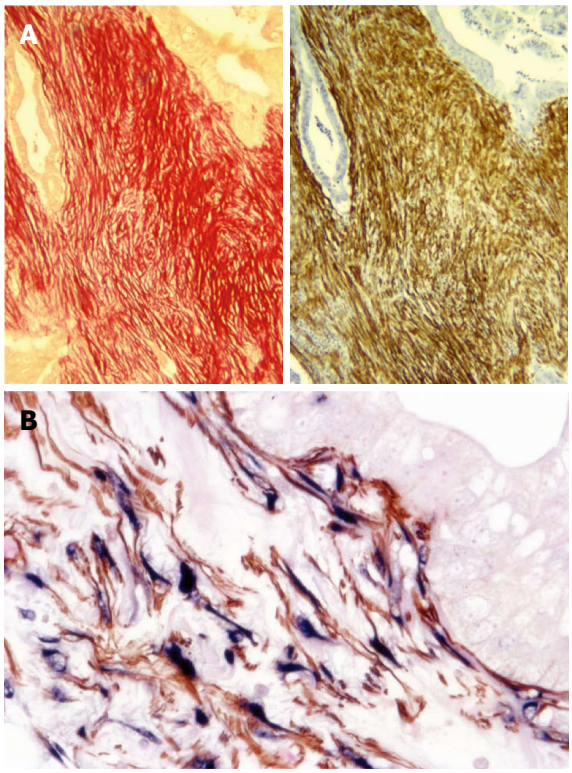

Figure 2 Pancreatic stellate cells are the source of collagen in stroma. A: A representative pair of serial sections of human pancreatic cancer tissue shows that Sirius Red staining for collagen (red) co-localises with immunohistochemical staining for α-smooth muscle actin (α-SMA) (brown), suggesting the presence of activated pancreatic stellate cells (PSCs) in the stroma of pancreatic cancer[33]. Reprinted with permission from *Wolters Kluwer Health* (Apte *et al*[33]); B: Immunohistochemistry for α-SMA (brown) and in situ hybridisation for procollagen α1 mRNA (blue), reveals colocalisation of α-SMA and procollagen mRNA on human pancreatic cancer tissue indicating that active PSCs are the major source of collagen in tumour stroma. Reprinted with permission from *Elsevier* (Apte *et al*[43]).

Collagen I promotes pancreatic cancer cell adhesion, proliferation and migration *via* integrin α2β1[35]. Collagen, fibronectin and laminin are also found to be associated with increased chemo-resistance of pancreatic cancer cells *in vitro*[36].

There is now unequivocal evidence that fibrosis of the pancreas is produced by pancreatic stellate cells (PSCs)[33]. PSCs were first isolated from rat pancreas in 1998 by Apte *et al*[37] using a density centrifugation method. A similar method to isolate human PSCs from histologically normal human pancreas was later described by the same group[38]. Bachem *et al*[39,40] reported isolation of human PSCs from fibrotic pancreatic tissue of patients with chronic pancreatitis[39] and pancreatic cancer[40] using an explant technique. With the availability of these methods to isolate and culture of PSCs, researchers have been able to make significant advances in the understanding PSC biology.

PSCs are resident cells of the pancreas and comprise about 4%-7% of total parenchymal cells in the gland[37,41]. There are abundant vitamin A containing lipid droplets in the cytoplasm, which is a marker of quiescent PSCs. PSCs synthesise the ECM proteins collagen, fibronectin and laminin. They also express the matrix metalloproteinases (MMPs), MMP2, 9 and 13 that degrade ECM and the tissue inhibitors of metalloproteinases (TIMPs), TIMP 1 and 2 that inhibit the activity of MMPs. Therefore, PSCs are thought to play an important role in maintaining a balance between ECM synthesis and degradation to maintain normal pancreatic architecture in health[41]. During pancreatic injury, PSCs are activated by factors such as ethanol (a known cause of chronic pancreatitis) and its metabolites, bacterial endotoxin, oxidant stress, cytokines and growth factors. Activated PSCs lose vitamin A droplets, assume a myofibroblast-like phenotype, express the cytoskeletal protein α-smooth muscle actin (α-SMA) and synthesise excessive amount of ECM proteins leading to fibrosis[37,41-44].

In a bid to shed some light on the differences between stellate cells in health and diseases, we have conducted microarray studies to examine differences in gene expression in human PSCs obtained from normal pancreas (benign pancreatic diseases: serous cystadenoma, *etc.*) *vs* the disease-activated PSCs isolated from chronic pancreatitis and pancreatic cancer tissue[45]. Multiple genes were found to be differentially expressed. Validation studies confirmed that MMP3 was upregulated 32.25 fold, collagen type Ⅳα1 (a basement membrane component) was downregulated 2.25 fold and syndecan-2 (a transmembrane heparan sulphate proteoglycan that plays a role in cell binding, cytoskeletal organization, migration and invasion[46]) was downregulated 2.04 fold. These three genes are postulated to be involved in ECM remodeling function and motility of PSCs. However, in depth characterisation of the role of these genes in the functional modulation of PSCs remains to be undertaken.

IDENTIFICATION OF PSCS AS SOURCE OF ECM DEPOSITION IN STROMA

Up until just under a decade ago, the prominent stroma/fibrosis in pancreatic cancer had been largely ignored. In 2004, Apte *et al*[33] demonstrated that PSCs produced the collagenous stroma in pancreatic cancer. Using serial sections of human pancreatic cancer tissue, the authors showed that the PSC activation marker α-SMA, co-localised with Sirius red stain for collagen (Figure 2A), as well as with PSC selective markers, desmin and glial fibrillary acidic protein. Most importantly, co-localization of staining for α-SMA and procollagen mRNA (using in situ hybridization) indicated that activated PSCs were the predominant source of the collagen in stroma (Figure 2B). The authors also found that conditioned medium from human pancreatic cancer cell lines increased the proliferation and activation of PSCs *in vitro*[33]. This was one of the first studies to initiate investigations into tumour stroma interactions in pancreatic cancer.

ROLE OF PSCS AS PROGENITOR CELLS

Recent evidence suggests that in addition to synthesising ECM proteins, PSCs may have other roles within the pan-

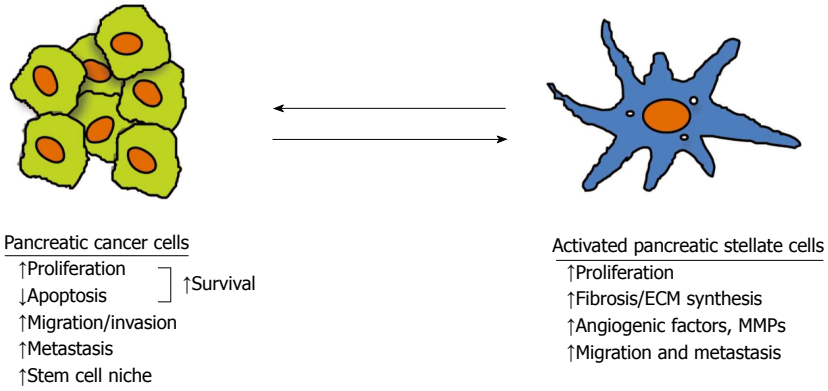

Figure 3 Interaction between pancreatic cancer cells and pancreatic stellate cells. Pancreatic cancer cells stimulate the proliferation, extracellular matrix (ECM) production, angiogenic factors and matrix metalloproteinase (MMP) expression, as well as migration of pancreatic stellate cells (PSCs); conversely PSCs increase proliferation and reduce apoptosis leading to increased survival, increase cancer cell migration and facilitate a cancer stem cell niche. The overall effect of interaction between pancreatic cancer cells and PSCs facilitates cancer progression[43].

creas, for example, as progenitor cells. In this regard, Mato *et al*[47] isolated and expanded pancreatic cells from lactating rats using mitoxantrone (a drug that acts through multidrug transporter systems) selection. They have reported that the surviving, mitoxantrone-resistant cells showed a PSC-like morphology (fibroblast-like with vitamin A lipid droplets), expressed the stem cell marker ABCG2 transporter (ATP binding cassette G2 transporter) and were able to secrete insulin after cell differentiation. More intriguingly, a recent study by Kordes *et al*[48] has reported that clonally expanded rat PSCs, when injected into hepatectomised recipient rats, are able to migrate to the liver and to reconstitute large parts of the liver by differentiating into hepatocytes and cholangiocytes, whereas muscle fibroblast do not show any such transformations.

IN VITRO INTERACTION BETWEEN PANCREATIC CANCER CELLS AND PSCS

In vitro studies assessing interactions between pancreatic cancer cells and PSCs usually involve co-culture experiments with these two types of cells (from humans and rodents) and/or exposure of one type of cell to the conditioned medium from the other. Pancreatic cancer cells have been shown to increase the proliferation and migration of PSCs as well as to induce the synthesis of collagen type I and fibronectin by PSCs[33,40,49]. Studies using neutralising antibodies have indicated that cancer cell-induced PSC proliferation and migration is mediated by platelet derived growth factor (PDGF), while the increase in synthesis of collagen I and fibronectin is modulated by the pro-fibrogenic factors, basic fibroblast growth factor (FGF-2) and TGF-β1 from cancer cells[33,40,49].

Other recently identified factors that may play a role in the interaction between cancer cells and PSCs include: (1) Cyclooxygenase (COX)-2, this enzyme is known to be constitutively expressed by PSCs. It catalyses reactions that transform arachidonic acid to prostaglandin H_2, the latter is then converted into prostaglandins, prostacyclin and thromboxanes to modulate inflammation, immune responses, mitogenesis, *etc*.[50]. Conditioned medium from cancer cells increases COX-2 expression and proliferation of PSCs. Mitogen-activated protein kinase kinase inhibitor, U0126, was shown to inhibit the cancer cell-induced increase in COX-2 expression in PSCs, while a COX-2 inhibitor, NS398, prevented cancer cell-stimulated PSCs proliferation, suggesting a role for COX-2 in cancer cell - PSC interactions[51]; and (2) Trefoil factors (TFF), a family of proteins secreted by the gastrointestinal mucosa, play a role in restitution after mucosal damage[52,53]. TFF1 expression is elevated in PDAC tissue and is detected in the majority of pancreatic cancer cell lines. It stimulates PSC proliferation and migration, as well as cancer cell invasion. The receptor and downstream signalling pathway for TFF1 are yet to be identified. TFF2 is expressed in chronic pancreatitis and PDAC, and has been shown to stimulate cancer cell migration in transwell membrane or wound healing experimental settings[53]. TFF2 is postulated to act *via* the chemokine receptor type 4 (CXCR4, a receptor for stromal derived factor-1) that is also expressed by pancreatic cancer cell lines and PSCs. Interestingly, CXCR4 expression is elevated in PanINs and in PDAC and promotes cancer cell metastasis, growth and survival[52,54].

In parallel with the above described studies examining the influence of cancer cells on PSCs, researchers have been studying the effects of PSCs on cancer cells (Figure 3). Conditioned medium from PSCs has been shown to stimulate pancreatic cancer cell proliferation, and this effect is inhibited by pretreatment of the medium with a PDGF neutralising antibody. Since cancer cells express PDGF receptor, it is thought that PDGF in PSC conditioned medium mediates the observed effect[49]. Conditioned medium from PSCs also stimulates cancer cell migration, invasion and colony formation (again mediated by PDGF), but inhibits apoptosis, and increases resistance to chemotherapy and radiation. ERK1/2 and Akt kinases in cancer cells are known to increase after incubation with PSC conditioned medium[49,55], suggesting

that these pathways mediate the responses of cancer cells to PSC conditioned medium.

PSCs secrete a cell adhesion protein named periostin, which has been found to stimulate cancer cell growth and to confer resistance on the latter to serum starvation and hypoxia. Periostin may also act in an autocrine manner on PSCs themselves leading to increased collagen I and fibronectin production. Collagen I might subsequently perpetuate the PSCs activation. Notably, cancer cells have been shown to induce periostin expression, and modulate collagen I and fibronectin expression in PSCs thus creating a supportive microenvironment for the tumour[56].

Evidence is now accumulating to indicate that PSCs may also influence EMT in cancer cells. A recent study has reported that a subpopulation of pancreatic cancer cells express endoglin (CD105, a TGF-β co-receptor); upon co-culture with PSCs, the proportion of CD105 positive cancer cells increases, and these cells exhibit a greater increase in migration activity compared to CD105 negative cells. Interestingly, in the CD105 positive population of cancer cells, mRNA expression of E-cadherin (an epithelial cell marker) is suppressed while vimentin (a mesenchymal cell marker) is over expressed, indicating that CD105 expression is associated with EMT in cancer cells[57]. These results suggest that PSCs may induce EMT in pancreatic cancer cells. This concept is supported by another study. Using organotypic *in vitro* cultures (collagen I coated culture wells), Froeling *et al*[58] reported that co-culture of cancer cells and immortalised human PSCs (obtained from donor pancreas and transfected with retroviruses containing cDNA encoding human telomerase reverse transcriptase) in this system resulted in decreased E-cadherin expression and increased beta-catenin expression of cancer cells, again signifying a transformation of the cells to a more mesenchymal phenotype.

While, as described above, there is strong evidence that PSCs significantly influence cancer cell function, some doubts have been raised in a recent study as to whether all PSCs uniformly exert such effects. Ikenaga *et al*[59] have demonstrated the existence of functional heterogeneity among the PSC population. They have reported that a sub-population of PSCs which express CD10, a cell membrane associated MMP, exerts a more inductive effect on cancer cell invasion and proliferation than CD10 negative PSCs. Thus, this study suggests that human PSCs (isolated from pancreatic tissue of pancreatic cancer patients) may differ in their ability to affect cancer cells, and explain, at least in part, the heterogeneity observed in patients with regard to rate of disease progression.

Recurrence is a well recognised feature of pancreatic cancer and recent studies suggest that this may be related to a population of cancer stem cells (identified by expression of markers such as CD24, CD44 and CD133) that are resistant to treatment[43,57]. Interestingly, PSCs have been reported to increase the stem cell characteristics of cancer cells by inducing the expression of cancer stem cell-related genes ABCG2, Nestin and LIN28[60]. This surviving cancer stem cell niche may be an important factor in pancreatic cancer recurrence.

IN VIVO INTERACTION BETWEEN PANCREATIC CANCER CELLS AND PSCS

While the *in vitro* studies noted above provided robust data on the direct interaction between cancer cells and PSCs, *in vivo* studies are essential in terms of biological/whole organism relevance. In this regard, two earlier clinical studies have reported findings to support a role for the stroma in cancer progression. Watanabe *et al*[61] have reported that the presence of fibrotic foci (which the authors postulated as representing intratumoural fibroblast proliferation) was associated with shorter survival in advanced pancreatic cancer, while Erkan *et al*[62] have reported that high α-SMA/collagen ratios in tumours correlated with poor prognosis. However, as detailed below, most of the *in vivo* evidence in support of tumour-stromal interactions in pancreatic cancer comes from experimental studies using tumour xenografts and genetically engineered mouse models.

Using a subcutaneous mouse model of pancreatic cancer, wherein tumours were produced by injecting a suspension of pancreatic cancer cells, alone or in combination with PSCs, into the flanks of mice, Bachem *et al*[40] showed that mice injected with both cell types exhibited larger tumours than those injected with cancer cells alone. Histological assessment of tumours indicated that cancer cell proliferation and stromal content were both increased in the presence of PSCs, an effect that would contribute to the observed increase in tumour volume. However, subcutaneous xenograft models have obvious limitations, since they do not replicate the appropriate microenvironment, nor can they provide information on metastasis.

In 2008, two research groups used similar approaches involving injection of a mixture of pancreatic cancer cells (cell lines MiaPaCa-2 or BxPC-3) and human PSCs (either primary culture[49] or immortalised cells[55]) into mouse pancreas. The results from these studies demonstrated that co-injection of cancer cells and PSCs yielded larger tumours with higher cancer cell density revealed by cytokeratin staining, increased fibrosis as determined by Masson's trichrome staining and higher number of activated PSCs (increased α-SMA staining). The incidence of metastasis was also higher in the presence of PSCs in both studies. It is interesting to note that these facilitatory effects of PSCs on cancer progression are not restricted to the PSCs derived from resected pancreatic cancer tissue (*i.e.*, PSCs that have been exposed to cancer cells prior to isolation). Xu *et al*[63] have demonstrated that normal human PSCs (isolated from normal pancreas) exert a similar facilitatory effect on tumour growth and metastasis in an orthotopic mouse model of pancreatic cancer. These findings suggest that PSCs are relatively quick to acquire tumour inductive properties after a relatively short exposure to cancer cells, supporting the concept that cancer cells are highly efficient and effective at recruiting surrounding PSCs, so as to set up a conducive microenvironment (such as ECM) for their own growth. Indeed, direct effect on cancer cells of ECM (produced by PSCs) have been demonstrated by Scaife *et al*[64]. The authors assessed

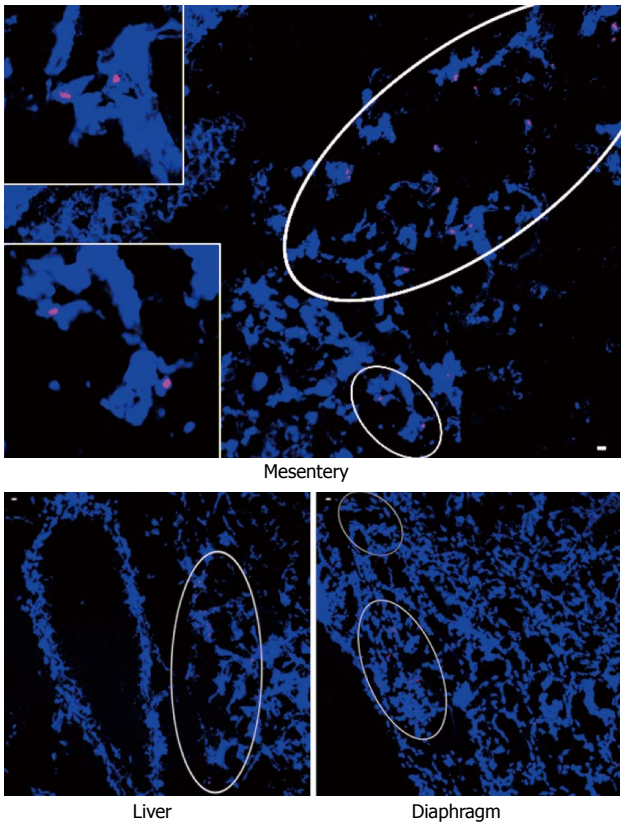

Figure 4 Identification of human pancreatic stellate cells from primary tumour in metastatic nodules. A representative photomicrograph showing Y chromosome positive cells in metastatic nodules in the mesentery (inserts are high power views of the circled regions), liver and diaphragm from mice (female) injected with female pancreatic cancer cells + male human pancreatic stellate cells, using fluorescent *in situ* hybridisation for the Y chromosome. Reprinted with permission from *Elsevier* (Xu *et al*[63]).

cancer progression in an orthotopic mouse model of pancreatic cancer by implanting a mixture of cancer cells and synthetic ECM (a hyaluronan-based hydrogel) into the pancreas of nude mice; the encapsulation of cancer cells within ECM yielded larger tumours than cancer cells suspended in serum-free media.

In contrast to orthotopic models where tumours are produced in immunocompromised mice by xenografts of human pancreatic cancer cells and PSCs, genetically engineered mouse models exhibit the development of spontaneous pancreatic tumours with a prominent endogenously produced stromal reaction. These models include KPC mice ($Kras^{LSL\text{-}G12D/+}$; $Trp53^{LSL\text{-}R172H/+}$; $Pdx^{cre/+}$), KPGC mice ($Kras^{LSL\text{-}G12D/+}$; $Trp53^{LSL\text{-}R172H/+}$; $R26^{LSL\text{-}GFP/+}$; $Pdx^{cre/+}$), and TGFβ type Ⅱ receptor organ specific knockout in the mouse pancreas ($Kras^{LSL\text{-}G12D/+}$; $TGF\beta r2^{floxflox}$; $Ptf1a^{cre/+}$). The tumours develop spontaneously with lesions progressing from preinvasive ductal changes to overt carcinoma and metastases. The stromal reaction also increases over time and importantly, activated PSCs are observed at the earliest time point (PanIN stages)[65-68]. It is anticipated that such models will be increasingly utilised to assess mechanisms of stromal-tumour interaction and new therapeutic strategies in pancreatic cancer.

ANGIOGENESIS IN PANCREATIC CANCER

Pancreatic cancer is poorly perfused, with the blood vessels in the tumours being often disorganised with irregular diameters, abnormal multiple branching, disrupted endothelial cells junctions and missing or disordered basement membrane. These changes cause the microvasculature of tumours to become leaky[69]. Angiogenesis is a complex process, and the response of endothelial cells in different parts of the tumour likely depends on the balance of the pro-angiogenic and anti-angiogenic factors within the surrounding microenvironment. It is possible that as activated PSCs lay down increasing fibrous stroma in central areas of the tumour, the blood vessels in that area are compressed, leading to insufficient perfusion and hypoxia. However, at the invading front of the tumour, where the collagenous stroma is significantly less dense, endothelial cell proliferation in response to activated PSC secretions can occur in a relatively unrestricted manner.

Masamune *et al*[70] reported that hypoxia induces migration, type I collagen expression, and VEGF production in PSCs. Interestingly, conditioned medium of hypoxia-treated PSCs induces endothelial cell proliferation, migration, and angiogenesis *in vitro*, possibly *via* VEGF. Xu *et al*[63] demonstrated that even when collected under normoxic conditions, conditioned medium from PSCs stimulated tube formation (a method of measuring angiogenesis *in vitro*) by human microvascular endothelial cells, and that this effect was again mediated by VEGF. Other factors produced by PSCs under hypoxic conditions include FGF-2, angiopoietin-1, periostin and hypoxia inducible factor-1[70,71], which may also promote angiogenesis.

ROLE OF PANCREATIC STELLATE CELLS IN PANCREATIC CANCER METASTASIS

Metastasis occurs early in pancreatic cancer, and has long been regarded as a feature solely exhibited by cancer cells. However, this concept has been challenged in recent time with studies indicating that untransformed epithelial cells[72] and mesenchymal cells[73] may also have the capacity to metastasise. It is now accepted that cells can travel to metastatic sites through the circulation either as single cells, or more likely, as a cluster of cells. We believe that metastatic cell clusters in pancreatic cancer could comprise both cancer cells and PSCs from the primary tumour. In this regard, Xu *et al*[63] used a gender mismatch approach (female pancreatic cancer cells plus male human PSCs injected into the pancreas of female mice) to demonstrate the presence of Y-chromosome positive cells in metastatic nodules (Figure 4). These results indicated that male PSCs from primary tumours were able to (1) intravasate into blood vessels; (2) be transported in the circulation; and (3) extravasate from blood vessels at metastatic sites. This possibility was supported by *in vitro* studies showing that PSCs can migrate through an endothelial cell monolayer *in vitro* and this transendothelial migration is up-regulated by PDGF from cancer cells.

In view of the above, we propose that PSCs that have travelled to the metastatic site perform a very important initial function at the metastatic sites, which is to facilitate seeding, survival and proliferation of the metastatic cancer cells at those sites. Also important is the likelihood that PSCs, *via* secretion of chemokines, subsequently recruit local stromal cells within the metastatic site, which further facilitates cancer cell growth.

IMMUNE CELLS, IMMUNE EVASION AND PSCS IN PANCREATIC CANCER

It is well established that pancreatic cancer tissue is infiltrated with immune cells, such as T cells, B cells, NK cells, neutrophils and macrophages[43,74,75]. Higher levels of $CD8^+$ T cell infiltration are correlated with a better survival[74,75], while macrophage and neutrophil infiltration show a negative correlation with survival[75]. Most recently, Ene-Obong *et al*[74] have reported that activated PSCs reduce the migration of CD8 positive T cell to cancer cells in human PDAC and the KPC mouse model of pancreatic cancer, indicating that PSCs may negatively modulate immune responses.

Immune evasion is also a characteristic of pancreatic cancer. Cancer cells have been shown to evade the host immune system by producing granulocte-macrophage colony-stimulating factor to suppress anti-tumour T cell immunity[76]. Recent studies suggest that PSCs may play a role in this process as well. PSCs in the stroma of PanIN lesions and around cancer cells produce galectin-1, a β-galactoside-binding protein[77,78], that binds to N-acetyllactosamine on membrane glycoproteins and induces apoptosis in T cells thus suppressing the immune response[79,80]. Fibroblast activation protein-α (FAP-α) known to be expressed by stromal cells, is another protein that has been reported to disrupt anti-tumour immunity. Depletion of the cells expressing FAP-α enabled immune response-associated tumour regression, supporting the notion that FAP-α might act as an immune suppressor in pancreatic cancer[81].

PANCREATIC STELLATE CELL AND ITS ROLE IN THE INITIATION OF NEOPLASIA

Evidence from recent studies is accumulating to indicate that PSCs might be activated at the earliest stages of carcinogenesis. Pandol *et al*[11] have found a distinct stromal reaction around PanIN lesions in a mouse model overexpressing $Kras^{G12D}$ that leads to pancreatic carcinogenesis. This stromal reaction is characterised by extensive collagen deposition and abundant α-SMA staining indicating the presence of activated PSCs around PanIN lesions. These findings corroborate those reported earlier by Fukushima *et al*[82] showing that periostin (solely expressed by PSCs) was observed in intraductal papillary mucinous neoplasms of the human pancreas. To assess the interaction between early neoplastic cells and PSCs, Pandol *et al*[11] isolated PanIN cells from the $Kras^{G12D}$ mice, and exposed PSCs to PanIN cell secretions. PSCs responded by increased proliferation, activation (α-SMA), fibronectin synthesis and MMP expression, indicating that preneoplastic cells have the capacity to activate PSCs in the early stages of carcinogenesis.

It is possible that in turn, these activated PSCs influence further progression of early lesions to established PDAC. In this regard, Funahashi *et al*[83] have reported that, nimesulide, a selective inhibitor of COX-2 (which as noted earlier is expressed by PSCs and implicated in PSC-cancer interactions[51]), retarded the progression of pancreatic cancer precursor lesions in a genetically engineered mouse model.

TARGETING THE STROMA OF PANCREATIC CANCER

The selection of treatment for pancreatic cancer patients depends on the stage of disease. The available options are surgery, chemotherapy, radiotherapy and recently developed targeted therapy, such as growth factor inhibition. Chemotherapy is the most frequently used treatment option for pancreatic cancer patients at different stages. Surgical resection with curative intent is only suitable for less than 20% of patients who have localised and early stage of pancreatic cancer[2,84]. Local recurrence is frequent and neoadjuvant and/or adjuvant therapies (chemotherapy and/or radiotherapy) are often required. For some advanced pancreatic cancers, surgery may be chosen to relieve obstruction and to improve the quality of life.

Gemcitabine was established as a first line chemotherapeutic drug for pancreatic cancer more than a decade ago, but it extends median overall survival only by several months[6]. Various combinations of chemotherapeutics have also been tried but regrettably the improvement has been negligible.

Based on an understanding of cancer cell biology and results from preclinical studies, several modalities targeting growth factor receptors and downstream signalling pathways have also been trialed. Unfortunately, these have not proved to be very successful. For example, the combination of erlotinib (an inhibitor of EGFR) and gemcitabine was shown to extend patient life by a mere two weeks *vs* gemcitabine alone. Other clinical trials involved inhibition of EGFR, VEGF and farnesyl-transferase by cetuximab, bevacizumab and tipifarnib respectively, but were not able to produce positive results[84]. The failure of translation of preclinical efficacy to the clinical situation may reflect the fact that many of the preclinical models used in these studies did not resemble human pancreatic cancer, in that, they lacked the stromal component.

In view of the above, it is clear that a comprehensive approach is needed to improve pancreatic cancer therapeutic efficacy. Given the increasingly recognised role of the stroma in cancer progression, there is a need to target not only cancer cells themselves but also the stromal elements in the tumour. The approaches discussed below have been built upon knowledge gained regarding PSC

biology and ECM composition.

The Hedgehog signalling pathway is thought to play an important role in PSC activation[85]. This pathway is crucial to embryonic development, and stem cell regulation in adults, but has also been implicated in tumour development. There are three Hedgehog ligands in mammals: Sonic, Indian and Desert Hedgehog[86]. This signalling pathway is inactive in health, and therefore not detectable in healthy adult pancreas[87]. In the absence of Hedgehog ligand, its cell membrane bound receptor, named Patched, represses another transmembrane protein, called Smoothened. The binding of Hedgehog ligand to Patched causes the repression of Smoothened to be lifted, leading to activation of downstream Gli proteins, a family of transcription factors that regulate genes related to cell functions such as cell differentiation, proliferation, apoptosis, adhesion and migration[67,88-90]. Abnormal activation of Hedgehog pathway has been shown in basal cell carcinoma, as well as lung, prostate, pancreatic cancer[89]; this activation can be Hedgehog ligand dependent (as in pancreatic cancer) or due to mutation of Patched (as in basal cell carcinoma)[91]. In pancreatic cancer, Smoothened was shown to be highly expressed by PSCs, and Sonic Hedgehog ligand to be expressed by pancreatic cancer cells only[88]. Feldmann *et al*[92] administered cyclopamine, a Smoothened antagonist, in a Pdx1-Cre;LsL-Kras^{G12D}; Ink4a/Arf$^{lox/lox}$ transgenic pancreatic cancer mouse model (crossbred LsL-Kras^{G12D}; Ink4a/Arf$^{lox/lox}$ and Pdx1-Cre; Ink4a/Arf$^{lox/lox}$) resulting in an extension of the overall median survival from 61 to 67 d. Olive *et al*[67] administered IPI-926, a semisynthetic derivative of cyclopamine, alone or in combination with gemcitabine in a KPC pancreatic cancer mouse model. IPI-926 binds to and inhibits Smoothened to keep Gli in an inactive form[91]. IPI-926 decreased collagen 1 content in stroma associated with a decrease in the proliferation of α-SMA positive stromal cells and transiently increased blood vessel density in primary tumours in KPC mice. The authors reported an improvement in delivery of chemotherapeutic agent to the tumours and an extension of the median survival from 11 to 25 d. Hwang *et al*[88] applied another Smoothened inhibitor AZD8542 in an orthotopic xenograft model of pancreatic cancer produced by a mixture of PSCs and cancer cells in different proportions (0:1, 1:1 or 3:1) and showed that AZD8542 significantly reduced tumour volume, lowered metastasis, decreased Hedgehog downstream signalling activity *via* decreased GLI 1 expression and increased tumour vascularity in tumours with a 3:1 proportion of PSCs to cancer cells. These studies imply that Sonic Hedgehog acts in a paracrine manner on stroma to facilitate pancreatic cancer progression, and that Hedgehog inhibition represents a potentially useful additional treatment approach for pancreatic cancer.

There are now several clinical trials targeting Sonic Hedgehog pathway inhibition in pancreatic cancer[6]. Unfortunately, despite encouraging results in phase I trials, the most recent phase II trial of gemcitabine and IPI-926 has resulted in a disappointing outcome. The trial had to be halted due to progressive disease and decreased median overall survival in pancreatic cancer patients treated with IPI-926 and gemcitabine[6]. The reasons for the failure of this drug in the clinical setting are not entirely clear. The disappointing clinical outcome may reflect the fact that: (1) results from a single preclinical model are not sufficient to account for the heterogeneity of human pancreatic cancer; and (2) the effects described by Olive *et al*[67] in the preclinical model, particularly with regard to perfusion, were transient. Before taking treatments to the clinic, it would be prudent to ensure that robust, long lasting effects were demonstrable in the preclinical setting.

Most recently, researchers have utilised other compounds to target the stroma of pancreatic cancer. Kozono *et al*[93] administered pirfenidone (a pyridone compound that has been shown to be an effective antifibrotic agent in idiopathic pulmonary fibrosis) in subcutaneous and orthotopic models of pancreatic cancer. The results revealed that pirfenidone decreased the growth of tumours produced by the injection of a mixture of pancreatic cancer cells and PSCs, but not the growth of tumours produced by cancer cells alone. *In vitro*, the authors found that pirfenidone inhibited PSC proliferation, invasion and migration, and interrupted the interaction between pancreatic cancer cells and PSCs. These effects of pirfenidone were associated with decreased expression of PDGF-A, hepatocyte growth factor, periostin, collagen type I and fibronectin in PSCs, as well as reduced PSC activation as evidenced by decreased α-SMA expression in the cells. The findings suggest that pirfenidone regulates PSC function and inhibits cancer growth.

Angiotensin inhibitors, used routinely for treatment of hypertension, have been suggested as a potentially effective treatment in pancreatic cancer[94]. Angiotensin Ⅱ is known to be able to stimulate PSC proliferation, migration, ECM production, and increase expression of FGF, TGF-β and VEGF[95,96]. Thus, angiotensin inhibition is postulated to prevent the activation of PSCs. Masamune *et al*[96] administered an angiotensin Ⅱ type I receptor blocker, olmesartan, in a subcutaneous mouse model of pancreatic cancer. Similar to the effect of pirfenidone, olmesartan only inhibited the growth of tumours produced by injection of pancreatic cancer cells with PSCs, but not that of the tumours produced by cancer cells alone. The authors also reported that olmesartan reduced α-SMA expression and collagen deposition in tumours and decreased PSC proliferation and collagen I production *in vitro*. Similar to the results with olmesartan, Chauhan *et al*[97] have reported that another angiotensin Ⅱ receptor inhibitor, losartan, decreased the density of α-SMA positive cells, collagen and hyaluronan production in the stroma of pancreatic cancer in an orthotopic mouse model. The effects of losartan might be mediated though reduction of TGF-β1, connective tissue growth factor and endothelin-1 (downstream target of TGF-β1) expression, all of which regulate ECM production by PSCs. The authors reported that the reduction in stroma decreased the physical pressure within the tumour, leading to improved perfusion and more effective drug delivery. The studies

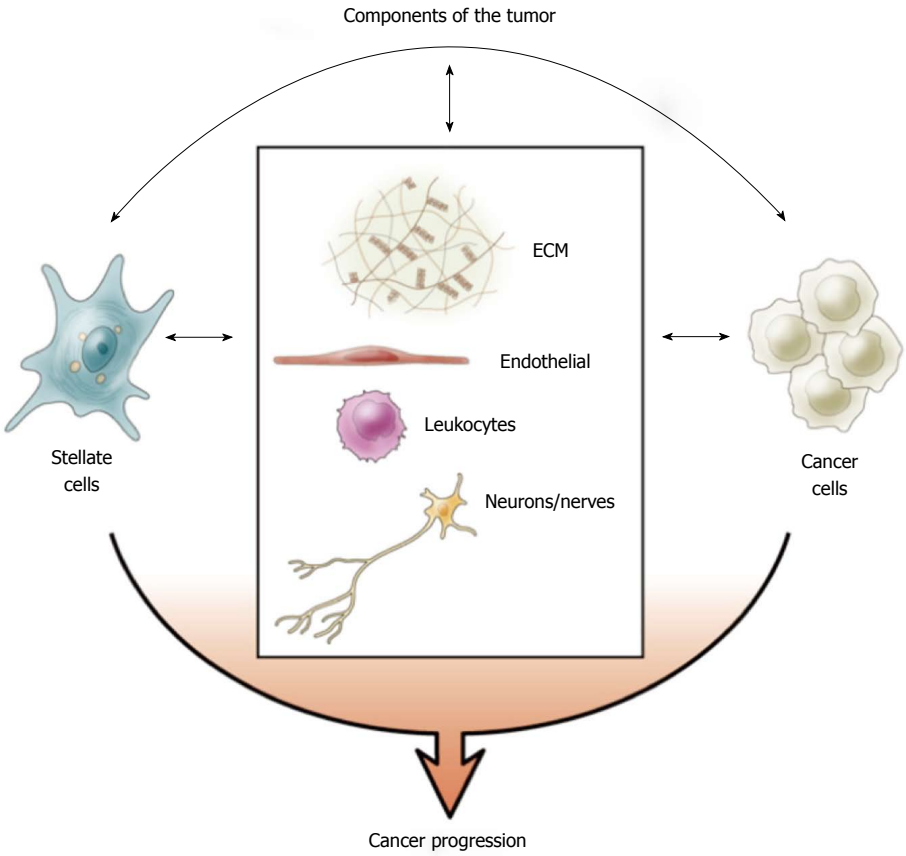

Figure 5 Tumour components. The stromal reaction of pancreatic ductal adenocarcinoma is comprised of pancreatic stellate cells (stromal cells), abundant extracellular matrix (ECM), blood vessels/endothelial cells, immune cells and nerves/neurons[43]. The interaction between cancer cells and the components of stroma facilitates cancer progression. Reprinted with permission from *Elsevier* (Apte *et al*[43]).

discussed above indicate that these compounds influence PSCs (stromal cells) function to inhibit tumour growth.

Taxanes [paclitaxel and docetaxel (semisynthetic analogue of paclitaxel)] have been widely used as chemotherapeutics for several cancers, such as breast, ovarian and non-small cell lung cancer. Paclitaxel inhibits the depolymerisation of microtubules in the cell and blocks cells in G2 and M phases resulting in cell death[98]. However, toxicity and insolubility in water of solvent-based paclitaxel significantly limit its clinical application. To overcome the toxicity of solvent, nab-paclitaxel was developed through homogenisation of paclitaxel and human serum albumin under high pressure to yield nano-particles about 130 nm in diameter. Paclitaxel is encapsulated by albumin in these nano-particles and becomes water soluble. As it is solvent free in comparison with parental compound, the toxicity of nab-paclitaxel is low and tolerated very well by pancreatic cancer patients. The albumin also enhances drug delivery through albumin facilitated receptor-mediated transcytosis[98].

Von Hoff *et al*[99] administered nab-paclitaxel alone or in combination with gemcitabine in a patient tumour-derived subcutaneous xenograft model. Nab-paclitaxel resulted in stromal depletion, increased the blood vessel diameter, increased expression of mNestin (an endothelial cell marker) in the tumour, and improved the delivery of gemcitabine. The mechanisms mediating these effects of nab-paclitaxel have not been fully elucidated. It has been observed that nab-paclitaxel accumulates in the proximity of tumour cells. Researchers have postulated that secreted protein acidic and rich in cysteine (SPARC), an albumin binding glycoprotein that is overexpressed in pancreatic cancer stroma[100], might contribute to the accumulation of nab-paclitaxel near tumour cells[98]. Analysis of SPARC expression in a clinical trial of gemcitabine and nab-paclitaxel combination has shown that high SPARC expression was correlated with significantly longer median overall survival compared to patients with low expression of SPARC[6]. Another mechanism that is also proposed to explain the synergistic effect of nab-paclitaxel and gemcitabine involves decreased metabolic inactivation of gemcitabine by cytidine deaminase which is destabilised by the increased production of reactive oxygen species in cancer cells following nab-paclitaxel administration[98]. The combination of nab-paclitaxel and gemcitabine is currently the subject of several ongoing clinical trials for locally advanced primary tumours and/or metastatic pancreatic cancer, as well as in neoadjuvant settings[6].

Two recent studies have directly targeted stromal ECM by using enzymes such as PEGylated human recombinant PH20 hyaluronidase (PEGPH20), to enzymatically degrade one of the predominant components of the ECM, hyaluronan. The authors reported that

PEGPH20 treatment led to stromal depletion, resulting in decompression of tumour vessels and an increase in tumour vascular patency without increasing vessel density. PEGPH20 also increased fenestrations in endothelia and interendothelial junction gaps that increased the permeability of the endothelium to macromolecules. Thus, the delivery of gemcitabine was improved with the PEGPH20 and gemcitabine combination significantly inhibiting tumour growth and extending the median survival of KPC mice from 15 to 28.5 d compared to gemcitabine alone in the study done by Jacobetz *et al*[34] or 55.5-91.5 d in the study done by Provenzano *et al*[68].

Researchers have recently also turned their attention to immune cells in pancreatic cancer stroma. CD40 is a member of the TNF receptor family and plays an important role in the development of anti-tumour T cell immunity. Beatty *et al*[101] performed studies on the KPC mouse pancreatic cancer model showing that the administration of CD40 agonist antibody activated macrophages, induced caspase-3 expression (an indicator of apoptosis) and decreased collagen I content in tumours. The treatment of CD40 agonist antibody alone or in combination with gemcitabine induced a similar rate (30%) of tumour regression. This regression appeared not to be related to $CD3^+$, $CD4^+$ and $CD8^+$ T cells in this *in vivo* study. The data from a clinical trial reported by the same group showed therapeutic efficacy of gemcitabine and CD40 agonist antibody on metastatic pancreatic cancer[101].

In summary, both *in vitro* and *in vivo* studies have clearly demonstrated a critical role of the stroma in the pathobiology of pancreatic cancer. PSCs interact closely with cancer cells to modulate cell proliferation, ECM production, migration and invasion of cancer cells. PSCs also play an important role in immune evasion, chemoresistance, angiogenesis and recurrence of pancreatic cancer (Figure 5). It is now increasingly clear that targeting tumour cells alone is insufficient to improve pancreatic cancer clinical outcome. Results from preclinical models and recent (albeit early) clinical trials provide vital evidence to support the concept that a comprehensive and combinatorial approach targeting both the cancer cells and stromal components in pancreatic cancer may represent the treatment strategy required to significantly improve the clinical outcome of this devastating disease.

ACKNOWLEDGEMENTS

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P- Reviewer: Erkan M, Jaster R, Satoh K **S- Editor**: Zhai HH
L- Editor: A **E- Editor**: Wang CH

World Journal of Gastroenterology

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DOI: 10.3748/wjg.v20.i32.11230

World J Gastroenterol 2014 August 28; 20(32): 11230-11240
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (14): Pancreatic cancer

Strategies for early detection of resectable pancreatic cancer

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Received: November 1, 2013 Revised: March 4, 2014
Accepted: April 15, 2014
Published online: August 28, 2014

Abstract

Pancreatic cancer is difficult to diagnose at an early stage and generally has a poor prognosis. Surgical resection is the only potentially curative treatment for pancreatic carcinoma. To improve the prognosis of this disease, it is essential to detect tumors at early stages, when they are resectable. The optimal approach to screening for early pancreatic neoplasia has not been established. The International Cancer of the Pancreas Screening Consortium has recently finalized several recommendations regarding the management of patients who are at an increased risk of familial pancreatic cancer. In addition, there have been notable advances in research on serum markers, tissue markers, gene signatures, and genomic targets of pancreatic cancer. To date, however, no biomarkers have been established in the clinical setting. Advancements in imaging modalities touch all aspects of the clinical management of pancreatic diseases, including the early detection of pancreatic masses, their characterization, and evaluations of tumor resectability. This article reviews strategies for screening high-risk groups, biomarkers, and current advances in imaging modalities for the early detection of resectable pancreatic cancer.

Key words: Early detection; Resectable; Pancreatic cancer; Screening; High-risk group; Biomarker; Imaging

Core tip: To improve the prognosis of patients with pancreatic cancer, it is essential to detect tumors at early stages, when they are resectable. The cancer of the pancreas screening program has reached several conclusions and recommendations for the management of patients who are at an increased risk of familial pancreatic cancer. Furthermore, genetic, epigenetic, and proteomics research have improved the understanding of the mechanisms of this disease, potentially offering biomarkers that could allow the cancer to be detected early. This article reviews strategies for the early detection of resectable pancreatic cancer.

Okano K, Suzuki Y. Strategies for early detection of resectable pancreatic cancer. *World J Gastroenterol* 2014; 20(32): 11230-11240 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11230.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11230

INTRODUCTION

Pancreatic cancer is an especially lethal malignancy, with a mortality rate that almost equals its incidence. After pancreatic cancer is diagnosed, the 1-year relative survival rate is only 24%, and the 5-year overall survival rate is only 5%[1,2]. However, rates of overall survival have been improving over the past decades, for both resected and non-resected cases[1]. These improvements are believed to have resulted from more optimal patient selection, refinements in surgical techniques, and better postoperative patient care, in addition to the development of effective adjuvant therapies. In cases of pancreatic carcinoma,

complete surgical resection with adjuvant chemotherapy offers the best outcomes[3]. However, over 80% of patients with pancreatic cancer present with an unresectable primary tumor and distant metastasis at the time of diagnosis[4]. Of patients with resectable pancreatic cancers, only 15% have earliest-stage cancers (T1 or T2 tumors without lymph node metastases), which are associated with better survival[5,6]. Thus, only 2%-3% of all patients diagnosed with pancreatic cancer present with earliest-stage cancer. Among the patients with pancreatic cancer who undergo surgical resection, the 5-year survival rate is 15%-40%[7]. In a study of operated pancreatic cancers from the Japanese Pancreatic Cancer Registry, it was observed that patients with stage I tumors < 2 cm in size had considerably better survival (58% alive at 5 years) than patients with stage Ⅱb tumors (17% alive at 5 years)[1]. In another study, 100% 5-year survival was observed among 79 patients who had tumors < 1 cm and had undergone curative resection[8].

Recently, a valuable analysis about the timing of the genetic evolution of pancreatic cancer was reported[9]. The authors indicated at least a decade between the occurrence of the initiating mutation and the birth of the parental, non-metastatic founder cell. Furthermore, at least five more years are required for the acquisition of metastatic ability and patients die an average of two years thereafter. These data provide novel insights into the genetic features underlying pancreatic cancer progression and define a broad time window of opportunity for early detection to prevent deaths from metastatic disease. For these reasons, significant efforts have been invested towards identifying high-risk groups, sensitive biomarkers, and accurate imaging modalities for pancreatic cancer. Each of these advancements can facilitate the early diagnosis of pancreatic cancer that is resectable or potentially resectable.

CURRENT CRITERIA FOR RESECTABILITY

In the absence of metastatic disease, pancreatic cancer cases are classified into three main categories: resectable, borderline resectable, and unresectable. Recent revisions of the National Comprehensive Cancer Network (NCCN) guidelines have attempted to distinguish tumors that are clearly resectable from those that are borderline resectable[10]. Further, the NCCN guidelines provide a definition for radiographically resectable tumors. The specific NCCN guidelines have been quoted below[10].

Tumors considered "resectable" should demonstrate the following (1) No distant metastases; (2) No radiographic evidence of superior mesenteric vein (SMV) or portal vein (PV) distortion; and (3) Clear fat planes around the celiac axis, hepatic artery, and SMA.

Tumors considered "borderline resectable" include the following: (1) No distant metastases; (2) Venous involvement of the SMV or PV with distortion or narrowing of the vein or occlusion of the vein with suitable vessel proximal and distal, allowing safe resection and replacement; (3) Gastroduodenal artery encasement up to the hepatic artery with either short segment encasement or direct abutment of the hepatic artery, without extension to the celiac axis; and (4) Tumor abutment of the SMA not to exceed > 180° of the circumference of the vessel wall.

To improve the prognosis of patients with pancreatic cancer, it is essential to detect tumors at early stages, when they are more likely to be resectable.

SCREENING HIGH-RISK GROUPS TO FACILITATE EARLY DIAGNOSIS OF PANCREATIC CANCER

As presented in Table 1, previous studies have identified a variety of risk groups and factors for developing pancreatic cancer. An elevated risk of developing pancreatic cancer is associated with being a current smoker[11], African-American[4], over 55 years old[4], male[4], obese[12], previously diagnosed with intraductal papillary mucinous neoplasms (IPMNs)[13], or previously diagnosed with diabetes[12,14]. Additionally, family history can be used to identify some individuals who have a high risk of developing pancreatic cancer. An increased risk of pancreatic cancer has been linked to family histories of pancreatic cancer[15,16], chronic pancreatitis[17,18], hereditary pancreatitis[19,20], Peutz-Jeghers syndrome[21,22], familial atypical multiple mole melanoma, cystic fibrosis[23], and familial cancer syndromes, which include Lynch syndromes[24,25], familial adenomatous polyposis pAPC mutation, and hereditary breast and ovarian cancer syndrome with *BRCA1* and *BRCA2* mutations[26,27]. This section of our review focuses on screening guidelines, the importance of new-onset diabetes, and the identification of precancerous lesions for the early detection of resectable pancreatic cancer.

Screening programs

The cancer of the pancreas screening (CAPS) program is one of largest pancreatic screening initiatives to date. Results from the CAPS 1 and CAPS 2 studies show that early pancreatic neoplasia can be detected by screening asymptomatic patients[28,29]. In the CAPS 1 study, the diagnostic yield of screening was 5.3%. Most encouragingly, the patient who was diagnosed with pancreatic cancer as a consequence of screening is still alive and disease free more than 5 years after surgery[28]. CAPS 2 screening was performed using annual endoscopic ultrasound (EUS) and computed tomography (CT). Once an abnormality had been detected, endoscopic retrograde cholangiopancreatography (ERCP) was offered. Of the 72 high-risk patients, eight had pancreatic neoplasia confirmed by surgery or fine-needle aspiration biopsy (FNA), constituting a 10% yield of screening. The CAPS 3 study is an ongoing multicenter prospective controlled cohort study that involves annual screening using EUS and magnetic resonance cholangiopancreatography (MRCP).

Table 1 Risk factors for pancreatic cancer

Variables	Association	Ref.
Non-genetic risk factors		
Age	Ages 55-64 yr: 20.7% of cases; ages 65-74 yr: 25.8% of cases; ages 75-84 yr: 27.8% of cases; age 85 + yr: 13.3% of cases	[4]
Gender	The incidence rate is 13.8 per 100000 men and 10.8 per 100000 women	[4]
Smoking	Most established risk factor for PC. Risk increases significantly with greater intensity: ≥ 30 cigarettes/day (OR = 1.75, 95%CI: 1.27-22.42); duration ≥ 50 yr (OR = 2.13, 95%CI: 1.25-3.62); and cumulative smoking dose ≥ 40 pack-years (OR = 1.78, 95%CI: 1.35-2.34)	[11]
Obesity	Obese individuals (BMI ≥ 30) have a slightly higher risk (RR: 1.19) of developing PC compared with normal-weight individuals (BMI < 25)	[12]
Race	15.5 males and 12.6 females per 100000 in African-Americans, while 8.4 males and 6.9 females per 100000 for Asians/Pacific Islanders	[4]
Diabetes mellitus (DM)	Meta-analysis from 35 cohort studies revealed a RR ratio of 1.94 (95%CI: 1.66-62.27) between type 2 DM and PC. 40%-100% increases in the risk of PC are observed with established diabetes	[12,14]
New-onset diabetes	New-onset diabetes is associated with a four- to seven-fold increase in risk, such that 1%-2% of patients with recent-onset diabetes will develop PC within 3 yr	[30]
Intraductal papillary mucinous neoplasms	Standardized incidence ratio 16	[13]
Hereditary cancer syndromes		
Familal pancreatic cancer	1 first-degree relative: 4.6-fold increased risk (95%CI: 0.5-16.4); ≥ 2 first-degree relatives: 6.4-fold increased risk (95%CI: 1.8-16.4); ≥ first-degree relatives: 32-fold increased risk (95%CI: 10.2-74.7)	[15,16]
Chronic pancreatitis	An incidence ratio of 14-18 observed for the development of PC in CP cases, which is further increased by cigarette smoking	[17,18]
Hereditary pancreatitis	A 53-fold (95%CI: 23-105) increased risk for developing PC and a lifetime risk (age 70 yr) of PC of 30%-40% in comparison with normal. RR increases further in smokers	[19,20]
Peuts-Jeghers	132-fold (95%CI: 44-261) increased risk of PC compared with the general population	[21,22]
Lynch syndrome	8.6-fold (95%CI: 4.7-15.7) increased risk for developing PC compared with the general population. An estimated 3.68% (95%CI: 1.45%-45.88%) lifetime (age 70 yr) risk of PC	[24,25]
Hereditary breast and ovarian cancer	BRCA2 germline mutation carriers have a 5% lifetime risk of PC in comparison with 1.78% for controls. BRCA1 mutation is 2.26-times that of the normal population	[26,27]

PC: Pancreatic cancer.

CAPS 3 is also investigating magnetic resonance imaging (MRI) with secretin and a panel of candidate DNA and protein markers (in serum and pancreatic juice) as indicators of pancreatic neoplasms. Carbohydrate antigen 19-9 (CA19-9), macrophage inhibitory cytokine-1 (MIC-1), DNA hypermethylation, and *K-ras* gene mutations are presently under investigation as potential markers. The CAPS consortium has reached several conclusions and recommendations for the management of patients who are at an increased risk of familial pancreatic cancer[16]. The CAPS consortium specifically agreed that the following individuals were candidates for screening: first-degree relatives (FDRs) of patients with pancreatic cancer in a familial pancreatic cancer kindred with at least two affected FDRs; patients with Peutz-Jeghers syndrome; and carriers of *p16, BRCA2*, and hereditary non-polyposis colorectal cancer (HNPCC) mutations with at least one affected FDR. The consortium agreed that initial screening should include EUS, potentially with MRI or MRCP, but excluding CT and ERCP. The consortium did not agree on optimal screening modalities, intervals for follow-up imaging, or the use of EUS-FNA to evaluate cysts.

In general, screening was recommended for high-risk individuals. However, additional evidence is needed regarding the sensitivity and cost-effectiveness of screening, as well as the choice of management strategy for patients with lesions that are detected by screening.

New-onset diabetes

The CAPS approach does not contribute to the early detection of pancreatic cancers that have completely sporadic onsets. To identify early pancreatic cancers in sporadic groups, it may be possible to screen patients at the onset of diabetes mellitus. The new onset of diabetes mellitus is occasionally associated with pancreatic carcinoma that is otherwise clinically silent and, indeed, is potentially resectable[30]. A population-based cohort study of 2122 diabetic individuals identified 18 (0.8%) patients who developed diabetes at age 50 years or older and were diagnosed with pancreatic cancer in the next 3 years. In this cohort of individuals who were newly diagnosed with diabetes, the ratio of observed-to-expected pancreatic cancer incidence was 7.9 (95%CI: 4.7-12.5)[31].

Diabetes is highly prevalent in cases of pancreatic cancer, even for early-stage pancreatic cancers[32-36]. Specifically, 50% of patients with stage I or II pancreatic cancer had diabetes[37]. Tsuchiya *et al*[36] observed abnormal glucose tolerance in 61% of patients with small pancreatic cancers (≤ 2 cm). A study of especially small pancreatic cancers (< 10 mm) noted a 33% prevalence of diabetes[38]. Because diabetes arises in almost half of patients with pancreatic cancer, it is an attractive target for early pancreatic cancer screening.

Identification of precancerous lesions

Precancerous lesions are ideal targets for early identification because they can be treated before developing into invasive cancer. The majority of pancreatic masses treated by surgical resection are IPMNs, which have been increasingly recognized as precursors to pancreatic ductal adenocarcinoma[39]. Post-resection cure rates are very high for IPMN that does not have an associated infiltrating ductal pancreatic adenocarcinoma[40,41]. Pancreatic intraepithelial neoplasias (PanINs) are small neoplasms ($\leqslant$ 5 mm) that are mostly found in the head of the gland and are thought to be the most common precursor to invasive pancreatic ductal adenocarcinoma[39]. Most precancerous lesions (and especially PanINs) can only be identified reliably after surgical resection. Because many healthy individuals have low-grade PanINs that will never progress to clinically important neoplasms[42], markers are needed to help differentiate between neoplastic and non-neoplastic pancreatic lesions, as well as to indicate the presence of microscopic high-grade PanINs that might be suggestive of future pancreatic cancer risk.

The most challenging aspect of screening and surveillance programs is the management of asymptomatic pancreatic lesions that are detected by imaging tests. It is essential to have individualized decision-making within multidisciplinary programs and prospective research studies.

BIOMARKERS THAT FACILITATE EARLY DIAGNOSIS OF PANCREATIC CANCER

Biomarker screening is one possible approach for identifying these early lesions. To date, over 2000 studies of possible biomarkers have been published[43]. Yet, biomarkers for the detection of small pancreatic cancer have not been validated.

Serum markers

CA19-9 is a sialylated Lewis (a) antigen; it is a carbohydrate that is produced by exocrine epithelial cells and is normally absorbed onto erythrocyte surfaces. The measurement of CA19-9 levels has never been shown to be effective as a screening test for pancreatic cancer. In a study of 10162 asymptomatic individuals, abnormal CA19-9 levels were identified in only 18 (0.2%) persons[44]. Although this study used a variety of screening tests, only four pancreatic cancers (0.04%) were detected. Pleskow *et al*[45] performed one of the first studies that established CA19-9 as a promising biomarker in pancreatic cancer. In this study of 261 patients (including 54 with pancreatic cancer), the sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of CA19-9 were 70%, 87%, 59%, 92% and 84%, respectively. In addition, preoperative CA19-9 test levels constitute false positives in the setting of biliary obstruction, which is present in the majority of patients with pancreatic cancer and various benign conditions related to the pancreas and biliary tract[46]. There is some evidence that preoperative CA19-9 measurements can help determine whether a pancreatic cancer is resectable[47]. Maithel *et al*[48] reported a strong association between preoperative CA19-9 values and the identification of unresectable pancreatic cancer that could not be recognized on diagnostic imaging studies. They recommend staging laparoscopy for pancreatic cancers associated with CA19-9 levels that exceed 130 U/mL.

Carbohydrate antigens of mucin-1 (MUC-1) have been investigated as potential means of improving on the performance of CA19-9[49]. Yet, none of the assays used to detect MUC-1 carbohydrate epitopes have proven to be superior to CA19-9 measurements. PAM-4 can be used to detect MUC-1 proteins expressed in pancreatic cancer with a greater specificity than MUC-1 proteins expressed in other cancers[50]. Additionally, initial studies have shown that an enzyme-linked immunosorbent assay directed at detecting circulating MUC-1 epitopes is more sensitive and specific than CA19-9 for identifying patients with pancreatic cancer[50].

In a recent study, serum MIC-1 was determined to be more sensitive than CA19-9 as a marker of pancreatic cancer[51]. MIC-1 belongs to the transforming growth factor-β superfamily, which was first identified in the context of macrophage activation[52]. MIC-1 is overexpressed in pancreatic, colon, prostrate, breast, gastric, and several other types of cancers[53-55], and therefore, it may prove useful for diagnosing other cancers[56]. In an investigation of pancreatic cancer and MIC-1 levels, 90% of patients with resectable pancreatic cancer had MIC-1 levels that were more than 2 standard deviations greater than those in age-matched controls. By comparison, only 62% of patients with resectable pancreatic cancer had elevated CA19-9. Elevated MIC-1 was observed to be independent of TNM stage. Further, elevated MIC-1 was observed in six of seven patients who had T1 or T2 cancers, but elevated CA19-9 was observed in only two of these seven patients[57]. Based on these findings, serum MIC-1 may prove to be useful as a component of pancreatic screening protocols for detecting early stage pancreatic cancers in high-risk groups[28,58].

Proteomics

Proteomics approaches have also been employed in an attempt to identify protein markers of pancreatic cancer[59-62]. Several groups have identified protein fragments in serum using surface-enhanced laser desorption ionization, which appears to have found protein fragments that function as diagnostic makers at least as effectively as does serum CA19-9[63,64]. Pancreatic cancer proteins have also been identified in serum using matrix-associated laser desorption ionization, which is another mass spectrometry approach[65]. Proteomics studies have identified several important proteins that are associated with pancreatic tumorigenesis, including galectin-1, gelsolin, lumican, 14-3-3 protein sigma, cathepsin D, cofilin, moesin, and plectin-1[60,66,67]. Gelsolin and lumican were later tested in plasma, showing an 80% sensitivity and a 95% specificity

as a composite biomarker for separating early stage pancreatic cancer patients (stages Ⅰ and Ⅱ) from healthy controls and patients with chronic pancreatitis (*via* selected reaction-monitoring-based targeted proteomics assays)[68]. The application of proteomics to the study of pancreatic cancer is still in its early stages and remains challenging. Yet, despite being an emerging technology, proteomics has already provided fundamental information that has improved our understanding of this disease's mechanisms. Further, proteomics potentially offers solutions for the early detection of this cancer.

Genetic and epigenetic markers

K-ras mutations are present in up to 90% of pancreatic ductal adenocarcinomas[69,70]. Accordingly, *K-ras* mutants have been thoroughly investigated as markers of pancreatic adenocarcinoma. In addition to invasive pancreatic cancers, K-ras mutations also occur in patients with chronic pancreatitis, persons who smoke, and PanINs in patients who do not have pancreatic cancer[69]. Additionally, mutant K-ras is detected in the blood of patients with advanced-stage pancreatic cancers more commonly than it is detected in the blood of patients with less advanced pancreatic cancers[71,72].

TP53 mutations have been extensively investigated as possible diagnostic markers of a variety of cancers. In the case of invasive pancreatic cancer, however, such mutations do not normally occur until late in the neoplastic process. *TP53* gene mutations are found in 70% of invasive pancreatic ductal adenocarcinomas[73]. Mutations occur throughout the *TP53* gene, although several nucleotide hot spots have been identified, at which mutations are especially common[74].

The strategy of combining markers can optimize the diagnosis of pancreatic cancer through molecular examination[75]. In a study of a combined marker panel, the combination of methylated p16, mutant K-ras, and a functional yeast assay for *TP53* mutations was investigated[75]. The authors concluded that the presence of *TP53* mutations was the most specific. With improvements in the technology for detecting mutations, *TP53* mutations in pancreatic juice may underpin an effective diagnostic strategy.

Pancreatic cancer is both a genetic and an epigenetic disease[76,77]. Various genes are methylated as pancreatic cancer arises, and non-neoplastic pancreatic tissues rarely show methylation of these same genes. Genes that are methylated in the process of pancreatic cancer formation are p16[78], RELN[79], DAB1[79], ppENK[80], Cyclin D2[81], SOCS1[82], *SPARC*[83], TSLC1[84], and others[85,86]. Because the methylation of some of these genes can be detected through methylation-specific polymerase chain reaction, and because some of these genes are also highly expressed in pancreatic cancers, epigenetic markers may provide an opportunity for the early detection of pancreatic cancers.

Other potential markers

Promising biomarkers have also been established for predicting the effectiveness of chemotherapy and immune-based therapy. The human equilibrative nucleoside transporter (hENT1) protein transports gemcitabine into cells. In a prospective randomized trial (RTOG9704), hENT1 protein expression was associated with increased overall survival and disease-free survival in pancreatic cancer patients who received gemcitabine, but not in those who received fluorouracil. These findings are supported by preclinical data; the gemcitabine transporter hENT1 is therefore a molecular and mechanistically relevant predictive marker of benefit from gemcitabine in patients with resected pancreatic cancer[87]. In addition to hENT1, key determinants of gemcitabine cytotoxicity include the activities of deoxycytidine kinase (dCK). Indeed, high levels of hENT1 and dCK predict longer survival times in patients with pancreatic cancer who are treated with adjuvant gemcitabine[88].

Mesothelin is a glycoprotein expressed on normal mesothelial cells. It is overexpressed in several histologic types of tumors, including pancreatic adenocarcinomas. A soluble form of mesothelin has been detected in patients with ovarian cancer and malignant mesothelioma, and has been found to have prognostic value. Circulating mesothelin is also a useful biomarker for pancreatic cancer. Furthermore, mesothelin-specific T cells can be induced in patients with pancreatic cancer. This suggests that mesothelin is a potential target for immune-based intervention strategies in pancreatic cancer[89]. Although it is not yet clear how these markers specifically relate to the early diagnosis of pancreatic cancer, they may be clinically useful for treatment selection.

Investigations of pancreatic juice have involved both genetic and epigenetic markers for pancreatic cancer. To date, mutant K-ras, p53 mutations, DNA methylation alterations, mitochondrial DNA mutations, and other potential genetic and epigenetic markers have been investigated in pancreatic juice[75]. The MitoChip allows investigations of the mitochondrial genome. Early studies using this novel technology suggest that it can be used to detect mitochondrial mutations in pancreatic juice samples that are taken from patients with pancreatic cancer[90].

Genetic, epigenetic, and proteomics research have improved the understanding of the mechanisms of pancreatic cancer, potentially offering biomarkers that could allow its early detection. It is critically important to validate the utility of these biomarkers in clinical setting as soon as possible.

IMAGING FOR THE EARLY DIAGNOSIS OF PANCREATIC CANCER

Every aspect of the clinical management of pancreatic diseases is influenced by imaging studies. Specific examples include the early detection and characterization of pancreatic masses, the identification of anatomical variants, investigations of local and vascular involvement, the determination of perineural and lymphatic invasion, margin assessments, the detection of distant metastases,

and assessments of tumor resectabilty[91]. Because effective screening markers remain elusive, imaging remains the primary form of screening for cases of familial pancreatic cancer, in addition to its more routine use in the staging and management of pancreatic cancer[28,29,92-94]. Recently, imaging accuracy has been improving as a result of technological improvements. However, imaging still fails to detect many lesions that are under a centimeter in size.

EUS

In comparison with other approaches to imaging, EUS has been growing in popularity. Indeed, EUS offers a large variety of benefits. First, it can detect pancreatic lesions and intraductal papillary mucinous neoplasms that are less than a centimeter in size with a greater sensitivity than is offered by abdominal ultrasonography, CT, or MRI. Second, EUS accurately judges deep tumors. Third, EUS-guided FNA enables lesion biopsies and has an excellent diagnostic accuracy (92%)[95]. Fourth, EUS detects lymph node metastasis and vascular infiltration with greater sensitivities than are offered by CT imaging. More specifically, advancements in contrast-enhanced EUS technology could improve the characterization of vessels in the desired lesions, the accuracy of tumor staging, the accuracy of tumor follow-up, and differential diagnosis. Additionally, improvements in EUS elastography could advance real-time evaluations of tissue stiffness. Finally, hybrid imaging (such as CT/ultrasonography or CT/ultrasonography/MRI) may offer an opportunity to improve the detection and characterization of focal lesions[96].

For lesions < 2 cm, EUS is associated with a sensitivity and accuracy that approach 100%, as well as a specificity > 95%[97-100]. In an analysis of EUS-FNA for pancreatic lesions < 3 cm, Tadic *et al*[101] demonstrated a sensitivity, specificity, positive predictive value, negative predictive value, and diagnostic accuracy of 68%, 100%, 100%, 73%, and 83%, respectively. Based on these results, it appears that EUS has become quite capable of providing histological evidence, for which there is a great needed. Therefore, EUS should be performed wherever sufficient expertise is available.

Multi-detector CT

The resolution and diagnostic capabilities of CT scanners have improved to remarkable extents. Currently, 64-section thin-cut intravenous contrast-enhanced multi-detector CT (MDCT) is the tool of choice for radiological investigations. Scanning occurs in a sequence of phases: non-contrast, arterial, pancreatic parenchymal, and portal venous. Key features of MDCT are its rapid anatomic coverage and excellent spatial resolution[102]. When employed for the detection of pancreatic cancers, the sensitivity of CT ranges from 75% to 100%, and its specificity ranges from 70% to 100%[97,99,102-105]. Yet, for lesions ≤ 2 cm in size, this sensitivity diminishes to 68%-77%[97,103], with an accuracy of 77%[99].

The diagnosis of small pancreatic carcinoma is aided by findings of dilatation of the main pancreatic duct (MPD) and associated pancreatitis[106]. In the case of associated pancreatitis, a contrasting effect is evident between the areas of the pancreatic parenchyma proximal and distal to the site of the MPD obstruction[107,108].

MRI/MRCP

CT and MRI/MRCP are the primary investigations that are most commonly performed for the diagnosis and staging of pancreatic cancers. The choice between CT and MRI/MRCP is generally determined by the availability these individual modalities at medical centers, as well as the availability of the technical expertise that is necessary for interpreting and reporting their results. Fusari *et al*[109] found that, for the diagnosis of pancreatic cancer, MRCP offered a sensitivity, specificity, accuracy, positive predictive value, and negative predictive value of 100%, 88%, 98%, 97%, and 100%, respectively. They also found that MRCP, when evaluating the resectability of pancreatic carcinomas, offered a sensitivity, specificity, accuracy, positive predictive value, and negative predictive value of 88%, 100%, 90%, 100%, and 70%, respectively. As outlined by Miller *et al*[100], the addition of MRCP to CT can offer substantial benefits to tumor diagnosis and staging in several contexts. MRI's excellent contrast resolution is beneficial for detecting small tumors on gadolinium-enhanced fat-suppressed images.

PET

PET is a functional imaging modality that can detect metabolic alterations in tumors, which may precede notable morphological alterations. The radioactive tracer ^{18}F-fluorodeoxyglucose (FDG) has been used extensively in the PET imaging of malignant tumors. PET/CT can accurately detect small primary pancreatic lesions, distant metastases, and post-surgery recurrences. As a result of these capabilities, PET/CT has become increasingly important in the diagnosis and management of pancreatic cancer[110-112]. Elevated glucose metabolism has been found in the precursor lesions of pancreatic cancer, which suggests that there may be an opportunity to detect these changes using PET/CT, and thereby improve the timeliness of diagnosis and patient outcomes[113].

We have previously investigated the role of FDG-PET with dual-time point evaluation in cases of small pancreatic cancer[114]. When investigated using FDG-PET with dual-time point evaluation, all TS1 tumors (< 20 mm) had higher standardized uptake values in the delayed phase than in the early phase, which suggested that the lesions were malignant tumors. These results indicate that FDG-PET with dual-time point evaluation is a useful modality for diagnosing small pancreatic cancers.

A recent meta-analysis[115] regarding the detection of pancreatic carcinoma found a pooled sensitivity of 90.1% for PET-CT, which was substantially better than the 81.2% pooled sensitivity of EUS. However, PET-CT was also associated with a pooled specificity of 80.1%,

while EUS had a pooled specificity of 92.3%. These results are similar to the findings of two previously published reviews of the literature on the same topic[116,117]. The role of FDG-PET in the early detection and accurate staging of pancreatic cancer is controversial. We suggest that future research should definitely focus on the development of more specific PET tracers for pancreatic ductal adenocarcinoma.

CONCLUSION

Despite advancements in surgical techniques and adjuvant treatment, the prognosis of pancreatic cancer has only improved marginally over the past years. Future research should continue and expand recent investigations of screening for high-risk groups, sensitive biomarkers, and imaging modalities for the early diagnosis of resectable pancreatic cancer. Recent studies have successfully identified pre-invasive neoplasms using accurate pancreatic imaging tests. These advancements are encouraging. They attest to the importance of additional studies that are aimed at identifying individuals at a substantially increased risk of developing pancreatic neoplasia.

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P- Reviewer: Martignoni ME, Pazienza V **S- Editor**: Zhai HH
L- Editor: A **E- Editor**: Wang CH

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DOI: 10.3748/wjg.v20.i32.11241

World J Gastroenterol 2014 August 28; 20(32): 11241-11248
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (14): Pancreatic cancer

Advances in pancreatic cancer research: Moving towards early detection

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Supported by Youth Foundation of Shanghai Municipal Health Bureau (in part), No. 122; Young Teachers Program of Shanghai University, National Natural Science Foundation of China, No. 81302087; and Natural Science Foundation of Shanghai, No. 13ZR1457400
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Received: October 27, 2013 Revised: March 2, 2014
Accepted: June 2, 2014
Published online: August 28, 2014

Abstract

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal forms of cancer. Substantial progress has been made in the understanding of the biology of pancreatic cancer, and advances in patient management have been significant. However, most patients (nearly 80%) who present with locally advanced or metastatic disease have an extremely poor prognosis. Survival is better for those with malignant disease localized to the pancreas, because surgical resection at present offers the only chance of cure. Therefore, the early detection of pancreatic cancer may benefit patients with PDAC. However, its low rate of incidence and the limitations of current screening strategies make early detection difficult. Recent advances in the understanding of the pathogenesis of PDAC suggest that it is possible to detect PDAC in early stages and even identify precursor lesions. The presence of new-onset diabetes mellitus in the early phase of pancreatic cancer may provide clues for its early diagnosis. Advances in the identification of novel circulating biomarkers including serological signatures, autoantibodies, epigenetic markers, circulating tumor cells and microRNAs suggest that they can be used as potential tools for the screening of precursors and early stage PDAC in the future. However, proper screening strategies based on effective screening methodologies need to be tested for clinical application.

Key words: Pancreatic ductal adenocarcinoma; Early detection; Diabetes mellitus; Pancreatic cancer

Core tip: Because pancreatic cancer is usually detected at an advanced stage and there is a lack of treatment strategies for advanced disease, it remains one of the most lethal solid tumors. Genetic and epigenetic alterations, miRNAs and tumor microenvironment promote the development of pancreatic cancer from precursor lesions to localized disease and further to metastatic disease in several years. An effective screening strategy for pancreatic cancer is therefore needed. New-onset diabetes mellitus associated with pancreatic cancer and recently identified novel circulating biomarkers should be explored as potential screening markers.

He XY, Yuan YZ. Advances in pancreatic cancer research: Moving towards early detection. *World J Gastroenterol* 2014; 20(32): 11241-11248 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11241.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11241

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal forms of cancer. It is the 13th most commonly diagnosed cancer worldwide[1], and the eighth lead-

ing cause of cancer death. No early detection tests are available and most patients with localized disease have no recognizable symptoms or signs; as a result, most patients (80%-85%) are not diagnosed until late in the disease, when the cancer has metastasized to other organs[2,3]. Survival is better for those with malignant disease localized to the pancreas, because at present, surgical resection offers the only chance of cure. Because pancreatic cancer responds poorly to radiation and chemotherapy, and so far most of the targeted therapy agents have failed to show a substantial benefit, PDAC in the advanced stage is associated with an extremely poor prognosis[2,3]. Therefore, the early detection of PDAC has gained increasing attention with the aim of improving the outcomes of patients with this disease. Recent studies have improved our understanding of the pathogenesis of PDAC, the relationship between diabetes mellitus (DM) and PDAC, and the role of circulating biomarkers. The present review will discuss the possibility of using these data to detect PDAC in its early stage and propose future research directions.

RECENT ADVANCES IN THE UNDERSTANDING OF THE PATHOGENESIS OF PDAC

Despite the short lifespan of patients diagnosed with PDAC, the disease usually develops over a long period of time. Based on a genetic evolutionary model[4], it is estimated that 10-30 years are required from the initiating mutation until the patient's death. In this model, there are three critical periods in the genetic evolution of the disease: T1 is related to the formation of precursor lesions such as pancreatic intraepithelial neoplasia (PanIN) and lasts until the infiltrating carcinoma is first formed; T2 describes the period from that time until a metastatic subclone develops within the primary carcinoma; and T3 is the period of metastatic dissemination of that subclone until the patient's death. A conservative estimate of 11.7, 6.8 and 2.7 years per interval, respectively, has been reported[4].

The development of PDAC involves multiple steps. It may originate from four distinct precursors, *i.e.*, mucinous cystic neoplasm (MCN), intraductal papillary mucinous neoplasm (IPMN), PanIN, and the newly added intraductal tubular papillary neoplasms (ITPNs)[5]. All four harbor varying degrees of dysplasia and stepwise accumulation of genetic alterations, suggesting progression of these lesions from benign toward malignant neoplasms. MCNs have a characteristic ovarian-type stroma. Approximately one-third of MCNs are associated with invasive carcinoma of a ductal phenotype. IPMNs are recently established clinical entities with characteristic features of mucin hypersecretion and duct dilatation. Some IPMNs are associated with invasive carcinoma and IPMNs are recognized as precursors to pancreatic cancer. ITPNs are rare premalignant tumours, with a concomitant invasive component, and were included for the first time in the 2010 World Health Organization classification[5]. PanINs are microscopic proliferative lesions arising from any part of the pancreatic duct system. Low grade PanINs are commonly found in the pancreatic ducts of older individuals, whereas high grade PanINs, previously called carcinoma *in situ*/severe ductal dysplasia, may eventually give rise to invasive pancreatic cancer. PanINs are divided into four categories based on the degree of dysplasia (1A, 1B, 2, and 3). Appropriate clinical management is critical for patients with MCNs, IPMNs and PanINs. The progression from PanIN to invasive PDAC has been intensively studied and reviewed elsewhere[6]. The established model describes the stepwise progression of PanINs to PDAC through the accumulation of several important genetic aberrations. KRAS mutation may be the first step driving this progression, and it is detected in approximately 99% of PanIN-1 lesions[7]. In addition, to overcome oncogene-induced senescence, loss of function of CDKN2A, and genetic inactivation of TP53, SMAD4, and BRCA2 are also required for the development of PDAC[8]. Further investigation of these precursor lesions is expected to reduce the mortality from pancreatic cancer.

The development of PDAC involves multiple genes. In 2008, detailed, global, genomic analyses found that a large number of genetic alterations (an average of 63) affect a core set of 12 signaling pathways and processes that are each genetically altered in 67%-100% of cases of pancreatic cancer[9]. However, the pathway components that are altered in any individual tumor vary widely. The 12 core pathways[9] are apoptosis, DNA damage control, regulation of G1/S phase transition, Hedgehog signaling, homophilic cell adhesion, integrin signaling, c-Jun N-terminal kinase signaling, KRAS signaling, regulation of invasion, small GTPase-dependent signaling (other than KRAS), TGF-β signaling and Wnt/Notch signaling. In addition to the core pathways described above, more recent data indicate that chromatin regulation[10] and axon guidance[10,11] are additional cellular processes that play a crucial role in pancreatic cancer. The pathways involved in each patient often vary. This finding may help account for the heterogeneous nature of tumors and offer insights into why agents targeting a specific gene in a pathway rarely result in a therapeutic advantage in more than a minor percentage of patients. Therefore, identification of the core players of each pathway and the factors connecting them is important. In addition to whole genomic alterations, numerous investigators have focused on particular PDAC-associated genes through laboratory and patient studies. Our group demonstrated that Periostin[12], X chromosome-linked inhibitor of apoptosis (XIAP)-associated, factor 1 (XAF1, a novel XIAP modulator)[13], angiotensin-converting enzyme 2[14], bone morphogenetic protein-2[15], eEF1A2[16], L1 cell adhesion molecule (L1-CAM)[17], and DJ-1[18] are associated with PDAC proliferation, apoptosis, invasion and/or progression.

MicroRNAs (miRNAs) are a highly conserved family of 18-24-nucleotide RNA molecules that regulate the sta-

bility or translational efficiency of complementary target mRNAs. More than 20 miRNAs involved in pancreatic adenocarcinoma biology have been identified and shown to affect tumor growth, metastatic potential, and chemosensitivity[19]. Combinations of miRNAs can be used to differentiate between pancreatic adenocarcinoma and other pancreatic pathologies, as well as to assess prognosis. Manipulations of miRNAs can decrease the rate of growth or reinstall chemosensitivity to certain chemotherapeutic agents. The most extensively studied miRNA, as far as pancreatic cancer is concerned, is miR-21, which has been associated with cell proliferation, metastatic ability, decreased gemcitabine sensitivity, and poor overall survival (OS)[20-23]. Other PDAC associated miRNAs were discussed elsewhere. Recently, several other miRNAs (miR-196a[24,25], 130b[26], 92a[27], 198[28], 221[29,30], 23b[31], 29a[32,33]) were shown to play an important role in PDAC.

Tumors are complex tissues in which mutant cancer cells have conscripted and subverted normal cell types to serve as active collaborators in their neoplastic agenda. Recent studies have shown that PDAC is one of the most stroma-rich cancers. The tumor microenvironment surrounds most of the tumor mass and consists of a dynamic assortment of extracellular matrix components and non-neoplastic cells including fibroblastic, vascular and immune cells. Recent work has revealed that the PDAC stroma supports tumor growth and promotes metastasis, and simultaneously serves as a physical barrier to drug delivery[34]. Pancreatic stellate cells (PSCs) identified in 1998, have the ability to trans-differentiate from a "quiescent" retinoid/lipid storing phenotype in the normal pancreas to an "activated" a-smooth muscle actin producing myofibroblastic phenotype[35]. The activated PSCs produce the extracellular matrix proteins that comprise the pancreatic tumor stroma, to facilitate pancreatic cancer development[35]. Sonic hedgehog signaling has been shown to be restricted to the stromal compartment and enhance the desmoplastic reaction[36]. Findings[36] suggest that increased HIF-1α produced by hypoxic tumors triggers the desmoplastic reaction in pancreatic cancer.

The genetic evolutionary model of PDAC suggests a detection window of several years (T1 + T2) for this disease. The visualization of PDAC precursor lesions using currently available imaging methods is limited; therefore, the detection of precursors is difficult. Improving our understanding of the mechanisms of precursors and PDAC development may help identify tumor biomarkers for this disease.

DM AND PDAC

Because of the low incidence of PDAC in the general population, population-based screening is not recommended. It is more practical to screen individuals at increased risk for PC based on their family history or identifiable genetic predisposition, or patients with diseases known to increase the risk of pancreatic cancer, such as chronic pancreatitis and type Ⅱ DM. Patients with a family history of pancreatic cancer or mutation carriers (germline mutations in the *BRCA2*, *PALB2*, *p16*, *STK11*, *ATM*, *PRSS1* genes and Lynch syndrome or Peutz-Jeghers syndrome) should be screened for pancreatic cancer according to the recommendations of the International Cancer of the Pancreas Screening consortium[37]. Here, we will discuss the relationship between DM and PDAC and the possibility of using new-onset DM as a marker for the detection of PDAC.

Increasing evidence suggests that DM is related to PDAC. It is now recognized that although long-standing diabetes is an etiological factor for pancreatic cancer, new-onset diabetes is its manifestation[38-40]. Epidemiological investigations have found that long-term type 2 DM is associated with a 1.5-fold to 2.0-fold increase in the risk of pancreatic cancer[40]. The evidence suggesting that new-onset diabetes is the manifestation of PDAC, or in other words, caused by PDAC, is that: (1) new-onset diabetes is associated with a high prevalence of PDAC; (2) diabetes associated with pancreatic cancer is predominantly new-onset; (3) pancreatic cancer resection ameliorates diabetes; and (4) experimental data. A meta-analysis[41] conducted in 2005 that included 17 case-control and 19 cohort and nested case-control studies published between 1996 and 2005 demonstrated that the combined age-adjusted and sex-adjusted odds ratio (OR) for pancreatic cancer associated with diabetes was 1.8 (95%CI: 1.7-1.9) and was lower still (OR = 1.5) in patients with a ⩾ 5 year history of diabetes. In a pooled analysis[42] of 2192 patients with pancreatic cancer and 5113 cancer-free controls in three large case-control studies conducted in the United States, diabetes was associated with a 1.8-fold increase in the risk of pancreatic cancer (95%CI: 1.5-2.1). Risk estimates decreased as the number of years with diabetes increased[42]. A study from our group[43] included 1458 patients with PDAC and 1528 age-, sex- and sociodemographic matched controls and showed that compared with controls, patients with long-standing diabetes (⩾ 2-year duration) had a moderately increased risk of PDAC, with an OR (95%CI) of 2.11 (1.51-2.94). Interestingly, a significantly higher risk was observed among cases with new-onset DM (< 2-year duration), with an OR of 4.43 (3.44-5.72) compared to controls without DM. On the other hand, the reported prevalence of DM in pancreatic cancer varies from 23% to 75%, with the majority being new-onset[44]. Our data also showed that 44.7% of PDAC patients harbor DM, and almost 2/3 are new-onset[45]. Data from our group and other groups showed that resolution of DM after pancreatic resection occurs in 41%-57% of PDAC patients with new-onset DM, in contrast to most patients with longstanding DM, who remain diabetic postoperatively[44-46]. Recognition of new-onset diabetes as an early manifestation of pancreatic cancer could improve the detection of asymptomatic, early-stage pancreatic cancer[38].

The impact of DM on the long-term outcomes of patients with PDAC has also been intensively studied recently, although the results are controversial. In patients

with stage I -IV pancreatic cancers, DM does not confer a worse prognosis; in fact, diabetics have a statistically significantly superior median survival[47]. A retrospective study of 344 patients who underwent surgical resection of pancreatic cancers showed that perineural invasion was significantly more common in diabetics, with a poor OS[47]. A multi-institutional retrospective study[48] reported a shorter OS and disease-free survival (DFS) in patients with preoperative DM. By stratifying DM into different groups (long-term/new-onset pre-surgical diabetes, resolved/unresolved post-surgical diabetes), we found that the heterogynous DM groups have different impacts on PDAC outcomes: longstanding DM is predictive of poor postoperative DFS and OS, whereas postoperatively resolved new-onset DM is associated with longer DFS and OS[45].

There are several possible mechanisms to explain the effect of diabetes on promoting PDAC progression, including the cellular proliferative effects of hyperglycemia, hyperinsulinemia, abnormalities in insulin/IGF receptor pathways, oxidative stress and inflammatory responses[40]. A prospective nested case-control study that included 449 case patients and 982 control subjects showed that the highest and the lowest quintiles of HbA1c, insulin, and proinsulin were associated with an increased risk for pancreatic cancer. However, in mutually adjusted models, only circulating markers of peripheral insulin resistance (proinsulin), rather than hyperglycemia (HbA1c) or pancreatic β-cell dysfunction (insulin), were independently associated with pancreatic cancer risk[49]. By comparing the proteome of PDAC with and without DM, our previous study indicated that regenerating gene (REG) I α may be one of the connections between DM and PDAC[50]. The number of REG I α positive cancer cells was significantly higher in pancreatic cancer patients with diabetes (n = 38) than in subjects without diabetes. Overexpression of the REG I α protein in pancreatic cancer cell lines resulted in accelerated cell proliferation and consequently tumor growth, both *in vitro* and *in vivo*[50]. The IQ motif containing GTPase activating protein 1-exocyst axis is a growth factor- and nutrient-sensor that couples cell growth and division. It may function at the interface of cancer and diabetes[51].

Several preclinical and observational studies have shown that anti-diabetic medications may modify the risk of pancreatic cancer. A case-control study showed that diabetics treated with metformin had a significantly lower risk of pancreatic cancer (OR = 0.38; 95%CI: 0.22-0.69, P = 0.001)[52]. Metformin significantly decreased pancreatic cell growth[53]. These effects could be attributed to disruption of the crosstalk between insulin receptor and GPCR signaling[54], or up-regulation of miR-26a or other factors[53]. However, in a recent meta-analysis that included eleven studies, 1770 cases of pancreatic cancer in 730664 patients with DM were reported, indicating no significant association between metformin, insulin, or TZD use and risk of developing pancreatic cancer, and use of sulfonylureas was associated with a 70% increase in the risk of developing pancreatic cancer[55].

CIRCULATING BIOMARKERS FOR PDAC

Because PDAC develops over a long period of time and the curative response is significantly better in patients with early disease, an early diagnostic marker could positively impact the outcome of patients. Circulating biomarkers are always preferred over others because of their ease of collection and relatively noninvasive nature. The current standard serum marker, sialylated Lewis blood group antigen CA19-9, is widely used, but its use is limited to monitoring responses to therapy and not as a diagnostic marker because of its poor sensitivity (41%-86%) and specificity (33%-100%)[56]. CA19-9 can arise among patients with benign pancreaticobiliary disorders, notably cholestasis, and 5% to 10% of the population does not express Lewis antigens.

In the last two decades, many biomarkers have been tested for PDAC detection, some of which have higher specificity and sensitivity than CA19-9. These are new antibodies such as PAM4 recognizing MUC-1[57], soluble iC3b[58], REG4[59], serum phosphoproteins extracellular signal-regulated kinase (p-ERK1/2)[60], CEACAM1, a proliferation-inducing ligand[61], DJ-1[62], and laminin, gamma 2[63]. Further validation studies including a large number of cases are required for the clinical application of these biomarkers.

DJ-1 is up-regulated in 68.5% of PDAC specimens and correlates with tumor invasion and metastasis. The secretion of DJ-1 by tumor cells implies its potential as a biomarker[64]. Our data showed that the area under the curve (AUC) of serum DJ-1 is higher than that of CA 19-9 in certain patients with PDAC, and serum DJ-1 level also predicts poor patient outcome. Other groups confirmed our results[65] and showed the increase of DJ-1 in pancreatic juice[66].

The sensitivity and specificity of these biomarkers, which may be insufficient when used alone, can be improved by using them in combination or together with CA19-9. In a recent study[67], CA19.9 showed a better AUC in combination with SYCN, REG1B and AGR2 than when used alone. When analyzed in combination, three panels [CA19.9 + REG1B (AUC of 0.88), CA19.9 + SYCN + REG1B (AUC of 0.87) and CA19.9 + AGR2 + REG1B (AUC of 0.87)] showed a significantly better AUC (P < 0.05) than that of CA19.9 alone (AUC of 0.82). The superiority of the combination of biomarkers was also shown by our group and others[64,68].

Several recent reports showed that aberrant miRNA production is an early event in the development of PanIN lesions[69,70]. MiRNAs 21[71,72], 155[71], 16[73], 196a[73], 1290[74], 221[30], 375 (lower in PDAC)[30], and 18a[75] were identified by using miRNA expression profiling or other methods as potential biomarkers of PDAC alone or in combination with CA19-9 and each other[72,73]. A recent meta analysis of three blood based miRNA studies reported that the median specificity and sensitivity were 0.91 and 0.96, respectively[72]. Our group together with other centers[71] in China screened differentially expressed serum miRNAs with Illumina's sequencing by synthesis technology

using pooled serum samples followed by RT-qPCR validation of a large number of samples arranged in multiple stages in 97 PDAC cases and 158 age- and sex-matched cancer-free controls. We established 7 miRNA-based biomarker model (miR-20a, miR-21, miR-24, miR-25, miR-99a, miR-185, and miR-191) for PDAC diagnosis. These biomarkers had high sensitivity and specificity for distinguishing various stages of pancreatic cancer from cancer-free controls and also accurately discriminated pancreatic cancer patients from chronic pancreatitis patients (AUC = 0.993). In 26 stage I pancreatic cancer cases, the positive rate of pancreatic cancer detection by the 7 miRNA-based biomarker set was 96.2%, which was significantly higher than that of CA19-9 (46.2%) or CEA (30.8%) in the same sample set. In 48 stage Ⅱ pancreatic cancer cases, the positive rate was 91.7%, which was also higher than that of CA19-9 (62.5%) or CEA (31.3%). Although miRNA detection is not currently used as a criterion for PDAC diagnosis, recent investigations indicated that they may be promising biomarkers in the near future.

Circulating tumor cells (CTCs) are tumor cells that have acquired the ability to enter the circulatory system. Studies have reported the presence of CTCs in peripheral blood in 40%-100% of pancreatic cancer patients[76,77], and their potential as biomarkers of PDAC was demonstrated recently[76-83]. CTCs have the potential to provide a surrogate for "real-time biopsy" of tumor biological activity. However, as CTCs are extremely rare, both enrichment and sensitive methods of detection are required for their enumeration[79]. Recently, using a modular system with innovative features, EpCAM positive CTCs were isolated from PDAC patients at high purity (> 86%) and with excellent yields (mean = 53 CTCs per mL)[76]. However, the high cost and involved procedure associated with this system constitute an obstacle to its clinical application.

CONCLUSION

The early detection of pancreatic cancer may benefit patients with PDAC. The slow development and progression of pancreatic cancer are closely associated with the activation of oncogenes, inactivation of tumor suppressor genes, altered expression of miRNAs, and activated tumor microenvironment. Therefore, a better understanding of the pathogenesis of PDAC may help detect PDAC or PDAC precursors at the early stages of the disease. However, a low rate of PDAC incidence and the limitations of current screening strategies make early detection difficult. A cost-effective screening strategy is required. The association of DM with PDAC may provide clues for early diagnosis and assessment of the progression of PDAC. Advances in the identification of novel circulating biomarkers including serological signatures, autoantibodies, epigenetic markers, circulating tumor cells and miRNAs have provided potential tools for the early detection of PDAC. However, there are currently no prospective studies investigating screening methods for PDAC in patients with new-onset DM, and biomarkers useful for this purpose or their combinations remain unidentified. Therefore, further studies are required in this field of research.

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P- Reviewer: Chen YP, Hsu jt, Meister T, Kapan S, Kar P
S- Editor: Ma YJ **L- Editor**: Wang TQ **E- Editor**: Zhang DN

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DOI: 10.3748/wjg.v20.i32.11249

World J Gastroenterol 2014 August 28; 20(32): 11249-11261
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Multidisciplinary treatment of rectal cancer in 2014: Where are we going?

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Received: February 22, 2014 Revised: May 8, 2014
Accepted: May 25, 2014
Published online: August 28, 2014

Abstract

In the present review we discuss the recent developments and future directions in the multimodal treatment of locally advanced rectal cancer, with respect to staging and re-staging modalities, to the current role of neoadjuvant chemo-radiation and to the conservative and more limited surgical approaches based on tumour response after neoadjuvant combined therapy. When initial tumor staging is considered a high accuracy has been reported for T pre-treatment staging, while preoperative lymph node mapping is still suboptimal. With respect to tumour re-staging, all the current available modalities still present a limited accuracy, in particular in defining a complete response. The role of short *vs* long-course radiotherapy regimens as well as the optimal time of surgery are still unclear and under investigation by means of ongoing randomized trials. Observational management or local excision following tumour complete response are promising alternatives to total mesorectal excision, but need further evaluation, and their use outside of a clinical trial is not recommended. The preoperative selection of patients who will benefit from neoadjuvant radiotherapy or not, as well as the proper identification of a clinical complete tumour response after combined treatment modalities,will influence the future directions in the treatment of locally advanced rectal cancer.

Key words: Rectal cancer; Cancer staging; Cancer re-staging; Neoadjuvant chemoradiation; Response; Treatment; Local control; Local excision; Complete pathologic response; Rectal cancer surgery

Core tip: There is a growing interest in the possibility of the preoperative identification of locally advanced rectal cancer patients who will or will not benefit from a preoperative chemoradiotherapy. This review evaluates the role of current available imaging techniques in this decision process and critically analyzes the results and future scenarios of the more limited surgical or observational approaches. In particular, the new trends following a pathologic complete response (*i.e.*, local excision, wait and see approach) are discussed on the basis of randomized trials and meta-analyses which form the basis for present treatment recommendations.

Vignali A, De Nardi P. Multidisciplinary treatment of rectal cancer in 2014: Where are we going? *World J Gastroenterol* 2014; 20(32): 11249-11261 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11249.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11249

INTRODUCTION

The treatment of rectal cancer has dramatically changed over time. Evidence exists from literature that up to 70% of patients with non metastatic rectal cancer present themselves as T3 or node positive rectal cancer[1]. This finding implies that major efforts should be directed in the cure of advanced rectal cancer. Current strategies in the management of rectal cancer are moving toward a tailored approach which is based on preoperative staging results, in their accuracy in re-staging the patient, determining how the patients have responded to the therapy,

and thus having a pivotal role in the selection of the different therapeutical options that could be potentially offered to a patient with locally advanced rectal cancer[2-7]. This is extremely fascinating since it represents the result of continuous research in rectal cancer biology, preoperative staging, surgical strategies including laparoscopic approach of rectal cancer and trans-anal surgery. The main progress in rectal cancer treatment, however is represented by the understanding that the overall five-year survival improvement which has been reported in the last two decades, could be only sustained by a multimodal therapy approach. This term means an interdisciplinary cooperation between surgeons, oncologists, radiologists and radiotherapists.

The present paper is directed to evaluate the state of the art in the multidisciplinary treatment of locally advanced rectal cancer and to evaluate which are the possible future scenarios in the treatment of locally advanced rectal cancer.

RECTAL CANCER STAGING

Preoperative staging is a crucial step in the multidisciplinary approach to rectal cancer, and it could be considered the base for a tailored approach to the tumour. The decision to submit a patient to preoperative chemoradiation (CRT) is mainly based on staging. Other factors include tumour histology, location, patient's morbidity, patient's age and medical diseases. Digital rectal examination, widely used in the past, for primary staging, can give information about the fixation of the tumour, sphincter involvement, distance from anorectal ring as well as to the size of the tumour, however it has a limited accuracy in establishing the depth of invasion which is approximately 65%[8].

The most common imaging modalities currently used in preoperative staging of rectal cancer are endorectal ultrasound (ERUS), magnetic resonance imaging (MRI) positron emission tomography (PET) scan and computed tomography (CT). This latter imaging technique however is mainly used to rule-out the presence of systemic metastasis, and it will be not discussed in the present review.

Pre-operative staging

ERUS: Data emerging from recent meta-analyses and cohort studies, showed that ERUS has an overall accuracy for T staging which ranges from 80% to 95%. In particular, the highest accuracy and specificity have been observed in the evaluation of early rectal cancer and in the assessment of tumour extent into the layers of rectal wall[9-14].

With respect to nodal staging, ERUS is less accurate, tends to overstage the patients with a reported overall sensitivity and specificity of 55% and 78%, respectively[10,11]. In particular data from a recent meta-analysis including 35 studies reported a pooled sensitivity and specificity of about 75%[14]. The sole size criteria used in the past for pathological node identification (*i.e.*, nodule of 10 mm or larger are certainly malignant) could be partially responsible for this wide variability in results. Wang *et al*[15] demonstrated that the majority of involved nodes following rectal cancer surgery has a diameter smaller than 5 mm and thus other factors are now considered in the determination of the nature of the nodule, such as roundness, border irregularity and hypoechoic nature. Even with these improvements, an overall false negative rate of 20% has been recently reported by Beets in a review article on this issue[16]. Other factors are potentially responsible for the reported wide variability in staging accuracy of ERUS: different probes, different professional figures (radiologist, gastroenterologist, colorectal surgeon performing the examination) and long learning curve[9,17,18]. Moreover an operator dependence has been reported with an inter-observer variation of 10% to 15% for T staging[10].

MRI: MRI has been demonstrated to be superior in imaging of the more advanced tumours, when compared to ERUS[16,19-22]. Moreover, another advantage of MRI, when compared to ERUS is the relatively small learning curve for the interpretation of the images, which could be more easily interpreted by other radiologists and clinicians[16,19]. This finding is of pivotal role when considering the multidisciplinary approach to rectal cancer. The main advantages of MRI have been reported to its ability in the identification of the mesorectal fascia, in particular in the threatened circumferential margin (CRM), while other diagnostic tools have showed low to intermediate accuracy in the evaluation of tumour penetration and involvement of the mesorectal fascia[19,23,24]. This correlates to its high accuracy in the preoperative identification of patients at risk of incomplete surgical excision[11,16,19,24,25].

Data from the multicenter, multinational MERCURY study group have demonstrated that MRI has an accuracy of 91% in predicting a clear CRM[26]. Recently, a meta-analysis on the same issue, reported figures in term of specificity in the prediction of an involved CRM ranging between 73% and 100% confirming the high accuracy of MRI in predicting CRM involvement[27]. Prognostic MRI features also include cancer less than 5 mm beyond the muscularis propria, mesorectal fascia more than 1 mm from the advancing edge of the tumour and absence of extramural vascular invasion. These features have been recently proposed to select patients who potentially do not need preoperative CRT[28]. Using these criteria, patients who underwent surgery alone had a local recurrence rate of 3% and a 5-year survival rate of 85%[29]. Phased-array MRI represents a step forward, in particular when an accurate assessment of tumor encroachment of the CRM is needed[25].

Current guidelines indicate pelvic phased-array coil as the most reliable tool in order to provide the most detailed depiction of the rectal wall and surrounding structures[24,30,31]. On the other hand, despite the continuous improvement in MRI technology, the use of new morphologic and signal-related criteria for lymph-node evaluation[32] and the introduction of lymph-nodes specific

MRI contrast agents, such as nano-paramagnetic particles of iron oxide[33], the accuracy of MRI in the preoperative identification of pathological lymph-nodes is still unreliable. Beaumont in a recent review of the role of MRI on rectal cancer staging has reported an overall accuracy of 69% and a sensitivity of 77% in the pathological lymph-node preoperative evaluation[24].

Positron emission tomography-CT scan: Positron emission tomography (PET)-CT scan has been recently proposed to be added to MRI for initial rectal cancer staging. However its role in T staging is low, due to its relatively low spatial resolution that ranges between a 0.4 and 1.0 cm and poor anatomical details[34]. The aforementionded limits also apply in the research of lymph-nodes micrometastases. Recent reports show that the accuracy of FDG-PET-CT in the evaluation of lymph-nodes is similar to the one of MRI with a reported sensitivity of 72%, and a specificity of 95%[34-37]. Nevertheless, FDG PET/CT maintains a consolidated role in M staging for rectal cancer and in detecting lymph-node at distant site, especially in the paraortic nodes[34,35]. Some reports have shown FDG PET/CT to detect 30% more distant lesions compared with CT scan, mainly in liver and lungs[11,37,38].

Re-staging modalities

As seen for the primary staging of the tumor, the accuracy in the restaging process after CRT is of paramount importance when deciding in favour of a more limited surgical procedure or when a non operative approach is contemplated. Both these new trends, in fact, are contemplated in case of extensive tumor response.

ERUS: With respect to ypT stage, ERUS shows an accuracy which varies between 27% to 72% with a high tendency to overstage the patient[39,40]. Figures within 0% to 60% have been variously reported when the accuracy to correctly diagnose a T0 is considered[39,41-44]. This low accuracy has been attributed to the difficulties in discriminating cancerous mass from desmoplastic reaction, peritumoral vasculopathy and radio-induced overgrowth fibrosis. This latter feature is particular difficult to differentiate at ultrasound for its hypoechoic pattern[45].

Better results have been reported when lymph nodal involvement restaging is considered, with figures ranging between 39% and 83%, resulting in a mean value of 70%[38,39,42,44]. For this parameter, overstaging was only slightly more common than understaging (8%-39% *vs* 11%-28%) as emerged in a recent review on this issue[43]. A higher diagnostic accuracy in N staging has been reported when patients were re-evaluated after 7 wk from the completion of CRT, compared to the 4-6 wk used by the majority of the authors, probably due to a reduction in the radio-induced fibrosis[46].

With respect to the accuracy in predicting a pathologic complete response (PCR), figures in term of accuracy ranging between 0% and 50% have been reported by means of small series and cohort studies[43,45-47].

MRI: The role of MRI in the restaging process is still sub-optimal[48,49]. A recent paper evaluating data from 5 institutional prospectively-maintained database demonstrated, using a sophisticated statistical method, the poor accuracy of MRI in predicting ypT, ypN, as well as the inability to predict a PCR or to discriminate a T4 disease[50]. Kim *et al*[51] reported an accuracy of 50% and 65% for rectal wall invasion and nodal involvement, respectively. Moreover, data from an Italian study, reported an accuracy in correctly identifying a ypT0 after neo-adjuvant therapy of 77% and of 65% for ypN0[52]. Radio-induced fibrosis, ulceration and proctitis might be responsible of this lack of accuracy[41,51,52]. This is particularly true for lymph-nodes restaging in which fibrosis makes it difficult to differentiate a metastatic lymph node and irradiated lymph node changes. In particular, a change in a lymph node with or without metastasis after neoadjuvant CRT is assumed to be associated with metastasis, resulting in lymph node overstaging[51]. The technical evolution of the MRI imaging with the introduction of the diffusion weighted MRI, perfusion MRI, lymph-nodes specific MRI contrast agents, seems to improve the accuracy of the imaging technique, in particular in the proper identification of a T0 lesion, however more data are needed to validate the role of these new imaging modalities[33,53-55]. Nevertheless, the actual prediction of a complete pathologic response is within the range of 66% to 85%[51,56,57].

PET: Concerning the role of PET in tumour response to therapy, some authors have suggested the complementary role of these imaging techniques to the most used MRI, CT scan and ERUS[34,58]. A growing interest among the scientific community in the potentiality of FDG PET/CT in evaluating the response of neoadjuvant therapy in rectal cancer has been reported, due to the promising results of the technique[59]. However, there is an urgent need for the standardization of the criteria used to measure the response[34,60]. Results, from recent reports, in fact, showed a wide range of values in term of sensitivity and specificity of FDG-PET in predicting a response after neoadjuvant therapy, which varies from 45% to 100% and from 59% to 96%, respectively. These results are related to different time-intervals in which the response is evaluated, different cut-off values and criteria used to define the response[34,59-62]. Moreover, with respect to the relation between PET and PCR, controversial results have been reported using 18-FDG-PET/CT[60,63-65]. These findings should be ascribed to the limit of the FDG PET/CT which has a spatial resolution of 5 mm, and it is not able to detect small cluster of cells, potentially limiting at present time its role in the prediction of complete response following CRT.

Conclusion: Staging rectal cancer

In Table 1 are summarized the pro and cons of each imaging technique in the staging and restaging process of

Table 1 Pro and cons of each imaging technique in the staging/restaging process

	Staging		Restaging	
	Pro	Cons	Pro	Cons
ERUS	High accuracy and specificity for early rectal cancer (T)	Tends to overstage N Operator dependent Long learning curve	High accuracy for persistent lymph nodal involvement	Low accuracy for T restage
MRI	Ability to evaluate CRM Best tool to select patients for neoadjuvant treatment High accuracy in advanced tumors	Low accuracy for lymph-nodes involvement	Good prediction for CRM involvement	Poor accuracy in predicting ypT0 and ypN0
PET	Confirmation of M and N at distant sites	Low accuracy for T staging	Detection of progression at distant sites	Lack of standardization of the criteria used to assess the response

MRI: Magnetic resonance imaging; ERUS: Endorectal ultrasound; PET: Positron emission tomography; CRM: Circumferential margin.

rectal cancer. An high accuracy has been reported for T staging using ERUS and MRI, while, despite the continuous technical progress in preoperative staging, preoperative lymph node mapping is still suboptimal with a false negative rate of 20%. PET scan is a promising imaging tool, but more data are needed to confirm its accuracy. With respect to the restaging process of rectal cancer following neo-adjuvant chemo- or radio-therapy (CT-RT), all the current available modalities still present limited accuracy, in particular when an accurate definition of clinical complete response is required.

In our opinion, the complementariness of these diagnostic tools should be kept in mind by the multidisciplinary team to obtain the most reliable information on the state of the tumour.

CURRENT ROLE OF PREOPERATIVE CT-RT

The current standard protocol in United States and Europe in patients with locally advanced cancer (T3, or any N1) is neo-adjuvant combined modality therapy (chemotherapy plus radiotherapy) prior to surgery[2,4-7,66,67]. Different randomized studies with high level of evidence were responsible for the adoption of this consolidated strategy[68-72].

The German CAO/ARO/AIO-94 trial, the Dutch TME trial, the MRC CR0//NCIC-CTG-C016 all demonstrated in patients who underwent preoperative CT/RT, a significantly lower local recurrence (LR) rate, a decreased toxicity, and increased sphincter preservation rate when compared with patients who underwent postoperative chemotherapy or surgery alone, while no significant difference was observed with respect to overall survival rate[68-70]. In contrast, data coming from another randomized controlled trial (RCT) trial, in which 240 patients with locally advanced cancer were enrolled, showed no difference in term of acute and late toxicity between preoperative and postoperative CRT, while a higher rate of sphincter preservation has been reported in patients who underwent preoperative CT (68% *vs* 42%)[72].

Sphincer preservation seems to have increased over time in the last 15 years. However, the role of preoperative CRT in decreasing the rate of abdomino-perineal amputation of the rectum (APE), thus resulting in an increased rate of sphincter preservation is still unclear and debatable. This issue has been recently addressed by Gerard *et al*[73] who analyzed the results of 17 trials randomizing close to 10800 patients. In this elegant analysis, none of the studies tested was able to demonstrate a beneficial effect of neo-adjuvant treatment on the rate of sphincter preservation. Other factors, such as the acceptance of progressive smaller distal margins, advances in surgical technology such as staplers, improvement in surgical techniques as inter-sphincteric resection could be responsible for the observed increased sphincter-saving reported by literature[7,74,75]. Another controversial issue is the role of neoadjuvant CT-RT in the management of unresectable rectal cancer (*i.e.*, palpably fixed lesion involving adjacent organ or structures, not amenable for primary surgical resection) which represented 15% of all rectal cancer at presentation. Chemoradiation aims for tumor shrinkage to allow radical resection. Two RCT trials demonstrated a higher resectability rate when chemoradiation was compared to radiation alone with figures in the range of 80%-85% for CRT *vs* 68%-75% for RT alone[76,77]. Moreover, the effect of boosted radiotherapy alone *vs* conventional neoadjuvant CRT on resectability has been recently evaluated by Engineer *et al*[78] in another RCT trial in which 90 patients with advanced or unresectable rectal cancer were included. Escalated radiation dose was not associated to a higher resectability rate, while it resulted in an increased wound infection and delayed wound healing. On the other hand preoperative short-course radiation could represent a valid alternative to CRT in elderly patients with primary unresectable rectal cancer unfit for preoperative chemotherapy due to severe co-morbidities[79].

The importance of adding chemotherapy to preoperative radiation was stressed in EORTC RCT trial published in 2006 (European Organization for Research and Treatment of Cancer). More than 1000 patients with locally advanced rectal cancer were recruited. A significant reduction of local recurrence from 17.1% to 8.7% was observed when chemotherapy was preoperatively added to 45 Gray (Gy) radiation delivered over 25 fractions[80]. The current recommended chemotherapeutic agent to use with preoperative radiation is capecitabine[81]. At

present time there is no consensus on which preoperative CRT scheme should be used; short or long-course CRT. Long-course scheme (LCRTCT) is the treatment of choice in North America and Canada[66]. In Europe, the scheme used to deliver preoperative CRT varies from country to country and different recommendations come from a panel of experts representing the most important European societies[82]. A moderate consensus to use short-course regimen (SCRT) was achieved for cT3 any NM0 disease. Agreement was reached on either SCRT followed by immediate surgery or LCRTCT with delayed surgery in patients with no CRM involvement. Moreover in patients not candidate for chemotherapy, SCRT with delayed surgery is an option/alternative. LCRTCT was recommended in patients with CRM involvement at presentations and in any cT4 any NM0. In this decision process, MRI prognostic features play a key role, in particular in the assessment of CRM involvement[67,82,83].

The main advantages of LCCRT over SCRT are tumour regression and downsizing as reported in the Polish and Trans-Tasman Group RCT trials which compare the two schemes[84,85]. In the Polish trial, a 16% complete pathologic response was reported for LCCRT, while it was 1% in the SCRT. Similar results were reported by the Trans-Tasman Group trial (15% *vs* 1% CR). No statistical difference was observed with respect of local recurrence and overall survival rate and late toxicity in both studies. A better downstaging response after long-course CRT when compared to short-course was also observed when a 6 wk interval to surgery was considered[86,87]. Data coming from the ongoing Stockholm Ⅲ trial will further clarify this issue. In this trial, three different randomization arms are considered; LCRT without concomitant chemotherapy, SCRT with immediate surgery or SCRT with surgery delayed for up to 8 wk in order to assess which treatment arm is more favourable in term of tumour regression, local recurrence rate and reduced toxicity[88]. An interim analysis of the Stockholm Ⅲ trial recently published, showed a close to 10% PCR rate, when SCRT followed by delayed surgery was considered, while figures of 0.4% and 2% were reported, in case of SCRT followed by immediate surgery and LCRT alone, respectively. Moreover SCRT followed by immediate surgery resulted in a higher complication rate when compared to the other treatment arms[88,89]. The use of SCRT and delayed surgery (6-8 wk after the completion of the treatment) has been recently proposed in patients with non resectable rectal cancer (synchronous/distant metastases) with contraindication to long-course CRT. These are patients in whom tumour regression and downsizing would not improve resection or sphincter preservation[90]. The results from this small series including 46 patients, show that delayed surgery was performed in all but nine patients, and that a complete pathologic response was obtained in 8.7% of the patients. The SCRT was well tolerated in the majority of the patients. Only one patients died due to sepsis with fever and neutropenia.

SELECTIVE USE OF PREOPERATIVE RADIATION

The well-recognized benefits of RT or CRT, in term of reduced local recurrence, increase rate of sphincter saving procedures, however need to be balanced against the risk of increased faecal incontinence, genitourinary disorders, impaired sexual function and bowel disorders[14]. Moreover, there is evidence in literature that TME surgery alone in the absence of preoperative radiation leads to local recurrence rates less than 10% and in a overall survival rate equivalent to preoperative radiation plus total mesorectal excision of the rectum (TME)[71,85,91].

Based on these results, several authors have focused their efforts to better identify the patients who are at low risk of local recurrence, and ideally may not benefit from neoadjuvant therapy[58,92,93].

Data from a Spanish institutional retrospective series on a population of 152 consecutive preoperatively stage Ⅱ or Ⅲ rectal cancer patients who underwent surgery alone, identified threatened mesorectal fascia at preoperative staging as the only independent preoperative factor associated with a significantly higher risk of local recurrence with a median follow-up of 39 mo[94]. This prognostic role of CRM was also confirmed by a Natgegaal and Quirke[95] on more than 17500 rectal cancer patient. Moreover data coming from NCRI colorectal cancer study group on 1156 patients identified the histological involvement of the circumferential margin as a powerful predictor of local recurrence, distant metastasis and survival rate[96]. On the basis of this evidence, it has been advocated the crucial role of CRM in the preoperative assessment of rectal cancer, in the light of neoadjuvant treatment, also suggesting that its assessment is more informative in treatment planning than the T stage[96-100]. Guidelines from an European consensus conference on rectal cancer, suggest that surgery alone is indicate in the early cT3N0 in presence of a clear circumferential margin assessed by MRI, unless the tumour is located at the level of the levators[67,82]. More recently, in a prospective single centre study, in presence of a good-quality TME, radiotherapy has been reserved only in patients with threatened or involved mesorectal margin irrespectively of the nodal status, with no adverse effect on local recurrence[99]. Guidelines from ESMO and EURECCA collaborative group proposed to sub-categorize rectal tumours in different subgroups (favourable, intermediate "bad group", advanced "ugly group") based on MRI findings in order to define the extent of surgery and whether neo-adjuvant CRT is required[101,102].

In summary, a predicted clear CRM as well as the other prognostic MRI features proposed by Heald *et al*[28] and stressed by others[25,29,30,67] already mentioned in this review (*i.e.*, absence of vascular invasion, upper third rectal cancer, absence of extramesorectal lymph-adenopathy, absence of neural or vascular invasion) seem to be able to identify patients at low risk, who will potentially not

beneficiate of preoperative radio and chemo-therapy. However, large randomized studies with high level of evidence are needed to implement this strategy.

Conclusion: Current role of preoperative CT-RT

According to these findings, one option could be to give neo-adjuvant therapy in the majority of patients, irrespective from their nodal status, leading to an overtreatment and its related consequences. The other future scenario is to reserve preoperative CRT only to patients with threatened CRM. This strategy should be indicated only by weighting the risk of unnecessary treatment against the possibility that these patients would ultimately require postoperative chemo-radiation which has a higher toxicity and it is less effective in term of local control when compared to preoperative CRT. However, this hypothesis is still awaiting, since more RCT trials and long-term follow-up studies are needed.

Which strategy following neo-adjuvant therapy

Current guidelines from the American Society of Colorectal surgeons indicate that radical surgery by means of TME with or without sphincter saving or partial TME depending on tumour localization should be offered to all patients following neoadjuvant treatment[66]. However, in light of the significant response rate that can be achieved with preoperative therapy, we have to consider and critically analyze the current role and implications of the new strategies proposed.

The benefits of neoadjuvant CRT have been well documented and include, among the others, tumour downstaging and tumour sterilization (*i.e.*, pathologic complete response) defined as the absence of cancer cells on histological examination in the resected specimen following radical surgery. PCR has been reported in 8%-40% of the patients either in phase Ⅱ/Ⅲ trials as well as in non-randomized trials as emerged in two recently published meta-analysis on this subject[103-105]. Different factors have been identified to influence the occurrence of PCR, such as the timing of response assessment, indicating that a longer interval between the completion of neoadjuvant therapy and surgery compared to the standard 4-6 wk adopted in the past by the surgical community, could increase the rate of PCR[106-108]. Moreover additional radiotherapy or dose escalation[109,110], novel chemotherapeutics agents and additional chemotherapy after preoperative CRT and before surgical resection have been variously documented to be able to improve PCR[104-106,110,111]. Patients who achieve a PCR have a favourable prognosis, with very low local recurrence rate (0%-1%) and 5-year survival rates greater than 95%[104,105,112-115]. Moreover there is evidence in literature that the risk of lymph-node metastases among patients with pathologic complete response is considerably low and frequently less than 5%[104,112,113,116,117]. A recent large series form Ireland, including 276 patients showed that in patients down-staged as ypT0/T1 the risk of nodal metastases was 2.3%[118]. This is in contrast with less radiosensitive tumours re-staged as ypT2-T4 in which the risk of harbouring nodal metastasis could be as high as 29%-64%[117]. According to the aforementioned findings, PCR may indicate a subset of patients associated with good outcome, but still at risk of lymph node metastasis, even low. This latter point is of crucial importance, since these patients could potentially beneficiate of a less invasive approach, avoiding a surgical procedure which is associated with a significant morbidity and long-term sequelae in term of sexual, urinary dysfunction and fecal incontinence[91,119-121]. According to these principles, a wait and watch approach has been proposed by Habr-Gama *et al*[122] from Brasil in patients in whom a clinical complete response (CCR: *i.e.*, absence of clinically detectable residual tumour) after neo-adjuvant therapy has occurred. CCR rates in the international literature range from 10.9% to 38.7% as recently reported in a review paper by Glynne-Jones *et al*[123], who evaluate the role of non operative approach after CRT in 650 patients. The Brasilian group has the largest experience on the non-operative approach to rectal cancer in patients who had a clinical complete response after neoadjuvant CRT. In their historical series published in 1998, of 118 patients with advanced low rectal cancer who underwent neoadjuvant therapy, 36 (30.8%) achieved a CCR. In these patients an observational approach was chosen with no immediate surgery, but a local recurrence which required a salvage resection occurred in 8 (27%) patients within 3 to 14 mo. Local recurrence and survival rate, however were similar to that of the patients with a PCR at surgery with a mean follow-up of 36 mo[122]. More recently published studies from the same institution, reported an early tumour regrowth (within one year) in approximately 17% of the patients[124,125]. However all the patients were amenable to salvage surgery with R0 resection, and the three-year overall and disease-free survival rate for patients with a sustained CCR was 94% and 75% respectively, with a median follow-up of 53 mo[125]. Authors from academic Institution from Holland and preliminary results from a phase Ⅱ clinical trial from England have recently reported their experience on the non operative approach to rectal cancer in patients with a complete clinical response reporting similar results in term of recurrence and overall and disease free-survival[126,127]. On the other hand, other studies, mainly of retrospective nature with inherent limitations in term of response assessment which was not standardized, reported disappointing results in term of local recurrence with figures ranging from 21% to 83% when a non-operative management following preoperative RT or CRT was considered[128,129]. The reproducibility of the results obtained by Habr-Gama *et al*[122], for the scientific community would be of fundamental importance for the wide application of a non-operative approach in patients with a clinical complete response following neoadjuvant treatment. Different variables should be considered in the interpretation of current available results. Major drawbacks are the definition of CCR which has evolved the course of published studies, in particular for

the Habr-Gama group series, the retrospective nature in the majority of published series, the absence of standardization in the methods used for determining response both in term of clinical and imaging modalities, the size of the tumour at initial evaluation, and the follow-up protocols which have been changed over time as underlined by Solanki *et al*[130] in a review paper on non operative management of rectal cancer after preoperative CRT. Another matter of criticism and caution in considering a non-operative approach, is represented by the fact that CCR does not necessarily correlate with PCR. A poor 25% to 30% concordance between clinical and pathological response has been reported by means of a large retrospective study from Memorial Sloan-Kettering Cancer Center and a review paper by Glynne-Jones in which 38 trials reporting data on complete/partial clinical response were analyzed[131,132]. Lastly, the rationale of the wait and see approach is based on an optimal restaging process following neoadjuvant treatment, but at present time, despite continuous technical advances in imaging techniques both with respect to MRI and PET-CT, restaging is still sub-optimal[33,56,57,60,63-65]. In summary, the need for a standardization both in definition of clinical complete response as well as in the follow-up procedure are of paramount importance, and only recently an expert consensus article has defined both clinical and endoscopic findings for CCR standardization that will be useful for future studies and their interpretation[133].

ROLE OF LOCAL EXCISION AFTER PREOPERATIVE CRT

Conventional TME is considered the standard of care following preoperative CRT. However, in selected patients in whom a significant tumour regression has occurred after neoadjuvant therapy, another surgical option is now represented by local excision. Moreover local excision using different surgical techniques has been recently proposed as a restaging biopsy, since both mucosal and submucosal endoscopic biopsies following CRT as well as digital examination should not be considered reliable procedures in the determination of a clinical complete response[3,39,68,134,135]. The main issue with respect to this approach, independently from the technique adopted; *i.e.*, transanal with retractors, TEM (trans-anal endoscopic approach)[136] or trans anal mini invasive surgery (TAMIS)[137] lies in the fact that only the rectal wall harbouring the tumour is removed without appropriate lymph-node dissection. Nevertheless, the rationale of proposing a local excision in patients who had a major response after CRT is based on the observation that the risk of nodal metastases depends from ypT status[62,138-140]. Different retrospective papers, small single series, have analyzed the role of local excision after major response to complete response following CRT[140-149]. In a recent pooled analysis by Borschitz *et al*[140] in which 270 patients were included, a strict correlation between local recurrence and pathological staging was reported. LR rate was 0% for ypT0 and 2% for ypT1, while for ypT2 figures between 6% and 20% were reported. According to the high recurrence rate reported in ypT2, the role of TEM in this sub-group of patients is controversial and there is no consensus on its use, at present time[116,145,146]. A recently published prospective multicenter phase Ⅱ study from Italy in which 63 patients with major clinical response after CRT were enrolled, reported a 0% LR rate after a mean follow-up time of 36 mo in the 43 patients who were ypT0 or ypT1/tumor regression grade (TRG) 2. Twenty patients resulted ypT > 2 or TRG > 3 or had positive margin following local excision; 11 underwent a TME, while 9 refused a TME. Among these latter 9 patients, a 22% LR was observed[147]. Similarly, another surgical group from Italy, found no local recurrence rate or distant metastases in patients in whom a PCR has occurred[148]. The interpretation of these data, however, needs some caution due to heterogeneity in staging as well in neoadjuvant regimens, to the inclusion of patients with high-co-morbidities or unfit for major surgery. Moreover, median follow-up times in the majority of published series, ranged from 19 to 56 mo[140]. This follow-up period has to be considered relatively short, since it has been demonstrated that pelvic recurrence following local excision may occur even after 5 years[146,147]. Finally, not in all studies, a sub-categorization of ypT1, ypT2 (*i.e.*, G1-2, *vs* G3, absence *vs* presence of lymphovascular invasion) has been reported[140]. The presence of lymphovascular invasion, as well as a poor tumour differentiation are well-known prognostic factors for risk of nodal metastases after neoadjuvant chemotherapy followed by TME and local excision and should be taken into account when considering a trans-anal excision[115,118,150]. Another matter of debate is the fact that local excision following radiotherapy increases postoperative morbidity in particular wound dehiscence[142,149]. Perez *et al*[145] comparing patients having CRT and TEM *vs* TEM alone, reported a 70% wound dehiscence in patients who underwent CRT and TEM *vs* 23% in patients who underwent TEM alone. A significant higher 30-d readmission rate was also reported in the same series, in the CRT plus TEM *vs* TEM alone (43% *vs* 7%, $P = 0.02$). A higher wound complication rate in the CRT plus TEM was also reported by others[143]. However in the majority of cases, they were treated conservatively as outpatients. Moreover, preliminary data from ACOSOG Z6041 trial also showed that preoperative CRT followed by local excision either by conventional transanal technique or TEM resulted in a persistent anal pain in 9% of the patients[151]. TEM excision can cause alteration or disruption of the surgical planes, resulting in a high risk of APE when a salvage or radical surgery is considered[152-154]. The true morbidity of TEM, postoperative quality of life as well as the risk of APE in case of salvage surgery need further investigation. The current CARTS (chemoradiation therapy for rectal cancer in the distal rectum followed by organ-sparing trans-anal endoscopic microsurgery) multicentric trial still ongoing will probably further clarify this issue[155].

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P- Reviewer: Meshikhes AWN, Wang ZH **S- Editor**: Nan J
L- Editor: A **E- Editor**: Zhang DN

World Journal of Gastroenterology

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DOI: 10.3748/wjg.v20.i32.11262

World J Gastroenterol 2014 August 28; 20(32): 11262-11272
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Multimodality magnetic resonance imaging in hepatic encephalopathy: An update

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Supported by Grants from National Natural Science Foundation of China, Nos. 30700194, 81171313, 81322020 and 81230032 (to Zhang LJ); and Program for New Century Excellent Talents in University, No. NCET-12-0260 (to Zhang LJ)
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Received: October 26, 2013 Revised: January 29, 2014
Accepted: May 28, 2014
Published online: August 28, 2014

Abstract

Hepatic encephalopathy (HE) is a neuropsychiatric complication of cirrhosis or acute liver failure. Currently, HE is regarded as a continuous cognitive impairment ranging from the mildest stage, minimal HE to overt HE. Hyperammonaemia and neuroinflammation are two main underlying factors which contribute to the neurological alterations in HE. Both structural and functional impairments are found in the white mater and grey mater involved in HE. Although the investigations into HE pathophysiological mechanism are enormous, the exact pathophysiological causes underlying HE remain controversial. Multimodality magnetic resonance imaging (MRI) plays an important role in helping to understand the pathological process of HE. This paper reviews the up-to-date multimodality MRI methods and predominant findings in HE patients with a highlight of the increasingly important role of blood oxygen level dependent functional MRI.

Key words: Hepatic encephalopathy; Magnetic resonance imaging; Magnetic resonance spectroscopy; Diffusion weighted imaging; Default-mode network; Blood oxygenation level dependent functional magnetic resonance imaging

Core tip: Multimodality magnetic resonance (MR) imaging is an effective and feasible research tool to uncover the pathophysiological mechanism of hepatic encephalopathy (HE). Among these MR imaging techniques, functional MR imaging method can be the most promising tool for studying HE. Nevertheless, the combination of functional MR imaging and other advanced MR techniques can be helpful to understand HE in the future.

Zhang XD, Zhang LJ, Wu SY, Lu GM. Multimodality magnetic resonance imaging in hepatic encephalopathy: An update. *World J Gastroenterol* 2014; 20(32): 11262-11272 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11262.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11262

INTRODUCTION

Hepatic encephalopathy (HE) is a neuropsychiatric disorder in patients with cirrhosis or after porto-systemic shunt procedure with pathophysiological and structural alterations. HE manifestations consist of a wide spectrum of neuropsychiatric and cognitive impairments from subclinical disturbance to stupor and coma[1,2]. HE is viewed as a continuous spectrum ranging from minimal HE (MHE) to overt HE (OHE), rather than distinct stages[1]. HE is a reversible condition after successful liver transplantation as the best method to cure advanced

liver disease[3,4].

The identification of MHE remains a major challenge for clinicians, mainly due to the intrinsic shortcomings of psychometric tests because these tests are easily affected by the individual's age, education, and extemporaneous neural status. Critical flicker frequency (CFF) posses the advantages of both language independence and moderate diagnostic accuracy for MHE, thus it is recommended as an adjunct (but not replacement) to psychometric testing[5]. In addition, it is believed that MHE develops before major neuropathological destruction occurs[6]. Hence, reliable and objective imaging biomarkers are necessary to complement these clinical tests to help identify or diagnose MHE and monitor the effects of therapy. Fortunately, these can be provided by various advanced magnetic resonance (MR) imaging tools. Here we review the multimodality MR imaging used in HE diagnosis, clarification of its pathophysiological mechanism, and its follow-up, with focus on new utilities or findings to date.

CLASSIFICATION OF HE

Based on etiology and severity of HE, the Working Party at the 11th World Congresses of Gastroenterology held in Vienna in 1998 recommended the nomenclature and three types of HE[7]. Type A, HE related to acute liver failure; type B, HE related to porto-systemic bypass without intrinsic hepatocellular disease; and type C, HE related to cirrhosis and portal cirrhosis and portal hypertension or porto-systemic shunts. Type C HE can be classified into 3 sub-categories: episodic, chronic, and minimal HE. The properties of episodic HE are confusional syndrome, acute onset, and fluctuant severity. Chronic HE includes relapsing HE (frequent episodes of acute HE) and persistent HE (not reverse despite adequate treatment). MHE is the mildest form of HE. In recent years, the investigation into MHE has been attracting more and more attention.

MHE refers to encephalopathy associated with cirrhosis or porto-systemic shunts or even with extrahepatic portal venous obstruction, and manifests subtly abnormal cognitive and/or neurophysiologic functions without clinically overt symptoms of HE[1]. The prevalence of MHE is estimated to vary from 30% to 84%[6]. The diagnosis of MHE still lacks gold standard, although a battery of psychometric tests have been used to detect neurocognitive impairment. However, neuropsychological tests cannot provide information about the cerebral regions involved. Compared with clinical manifestations of OHE, such as personality change, disorientation, and consciousness disorders, which may contribute to the increased risk of death of cirrhotic patients, MHE impairs executive functioning, working memory and health-related quality of life[8-10]. It has shown that patients with MHE are more likely to get driving problems[11]. In addition, MHE or previous bouts of OHE can be used to predict the subsequent development of OHE as well[9,12].

PATHOGENESIS OF HE

One widely accepted hypothesis for HE is hyperammonemia[13-15]. Hyperammonemia leads to excess uptake of ammonia by astrocytes representing the principal target. Increased ammonia and glutamate are converted to form abundant glutamine under the catalysis of glutamine synthase, which contributes to increased osmotic pressure. Moreover, astrocyte swelling may not happen immediately due to the existence of osmotic-regulatory mechanisms by depletion of intracellular osmolytes until decompensation. As decompensation continues, astrocytes undergo morphologic changes leading to Alzheimer type 2 astrocytosis.

In fact, HE appears to be a much more complicated process that is related to multiple synergistic precipitating factors[16,17]. These factors mainly include hyponatraemia, proinflammatory cytokines, ammonia and benzodiazepines which may induce the insult of astrocyte osmotic balance. Astrocyte swelling and brain edema may be a common pathway in the pathogenesis of HE[14-17]. However, hyperammonaemia and neuroinflammation are two main underlying factors which contribute to the neurological alterations in HE, even without the presence of cerebral edema. In fact, it is now believed that cytotoxic edema does not play an important role in MHE[18]. Changes of multiple neurotransmitter systems can lead to impaired neuronal communication in HE. That process results in various cognitive and motor impairments in HE even before cerebral structural alterations happen[19,20].

APPLICATION AND INTERPRETATION OF MULTIMODALITY MR IMAGING IN HE

Structural MR findings

MR imaging is regarded as the very useful tool for providing a broad range of structural and functional assessments of HE. It aids more understanding of the pathophysiology of HE with the advanced techniques, although other imaging approaches, such as positron emission tomography (PET), also demonstrate direct evidence for the pathogenesis of severe liver disease and MHE[1,2]. In acute HE, characteristic diffuse cortical overt brain edema can be seen on T2-weighted and fluid-attenuated inversion recovery (FLAIR) sequences, while cortical restricted diffusion is presented as high signal intensity on diffusion weighted imaging (DWI) and low signal intensity on apparent diffusion coefficient (ADC) map which indicates cytotoxic edema (Figure 1). However, the involvement of parietal, frontal, temporal, or occipital cortex has been found as an uncommon finding[18,21]. On the other hand, as far as chronic HE is concerned, apart from the well-known high signal intensity on T1-weighted MR imaging in bilateral basal

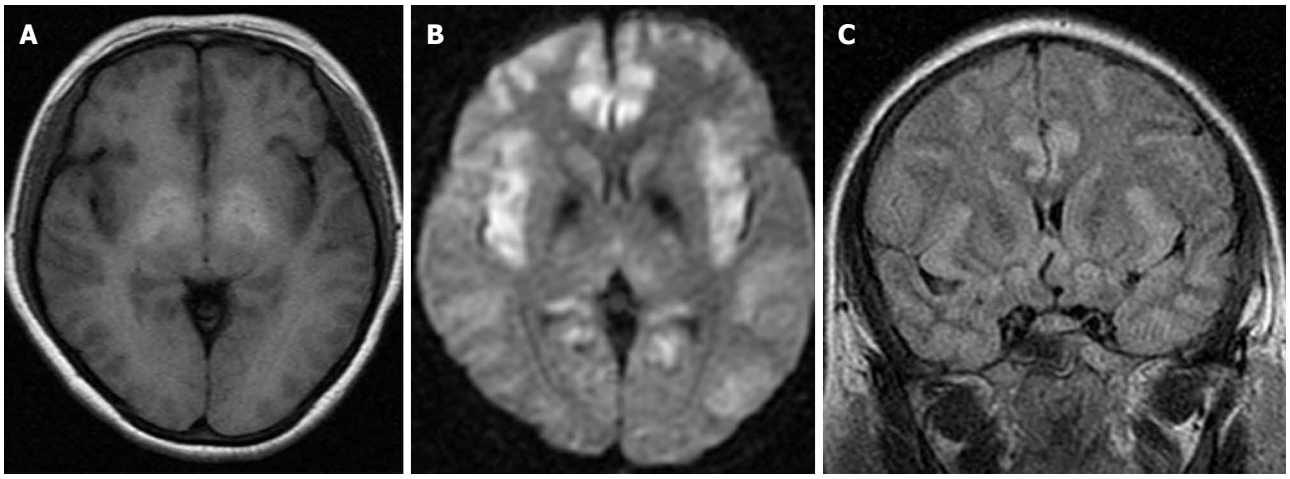

Figure 1 Acute hepatic encephalopathy in a 44-year-old female with hepatitis B virus-related cirrhosis. A: T1 weighted image shows high signal intensity of bilateral globus pallidus; B: T2 Fluid Attenuated Inversion Recovery image shows diffused cortical edema; C: Axial diffusion weighted image shows diffuse cortical high signal intensity corresponding to diffuse cortical edema.

ganglia, high signal intensity along the hemispheric white matter in or around the corticospinal tract on FLAIR T2-weighted images has been reported, which mimics the MR imaging findings of amyotrophic lateral sclerosis, due to mild brain edema beyond the threshold for detection on standard or conventional MR imaging.

Recently, some advanced MR analysis algorithms have been used to investigate brain structural changes in HE patients. Voxel-based morphometry (VBM) is a useful, unbiased and automatic research tool in accurately detecting the focal and global structural changes in both the grey matter and white matter. This method has been widely used in various central nervous system diseases[22]. Currently, a consensus has been reached as a decreased grey matter volume in cirrhosis patients. In a study by Guevara *et al*[23], a significant loss in brain density in many areas of the grey matter was found in patients with cirrhosis, which pointed to regional brain atrophy. Apart from the reduction of regional grey matter volume, Zhang *et al*[24] also found the increased thalamus volume in cirrhotic patients, which was not associated with HE progression, and multiple covariate regression results suggested that Child-Pugh score was a major factor to affect grey matter volume, while porto-systemic shunt mainly affected white matter volume. Recently, Tao *et al*[25] concluded that increased thalamic volume could be a potentially objective imaging biomarker for predicting seizures due to MHE with the area under the receiver operating characteristic curve of 0.827. Compared with cortical volume, cortical integrity has drawn less attention. Cortical thickness analysis with subvoxel resolution can be used to study cortical integrity in patients with MHE by Montoliu *et al*[26]. In their study, cortical surface-based analysis techniques were used to investigate patterns of cortical thinning in MHE patients. This technique is a kind of computational neuroanatomy analysis techniques applied to high-resolution cerebral MRI, which is helpful in detecting neocortical mantle. Finally, a focal thinning of the superior temporal cortex and precuneus in MHE patients compared with non-MHE patients and controls was found in this study. In terms of cortical morphological analysis, VBM techniques and cortical thickness analysis reveal signatures of the cortical mantle with different precisions, however, the former carries out a better matching of homologous cortical regions. In addition, white matter abnormalities are common in cirrhotic patients, and VBM is also used in measurement of white matter macroscopic alterations in HE patients. Guevara *et al*[23] found a loss of white matter density in patients with cirrhosis, thus, they considered that it might represent a loss of axons secondary to the loss of neurons. Zhang *et al*[24] found increased whiter matter volume, with the extent of affected brain volume greater in HE patients than in non-HE patients with cirrhosis. Moreover, white matter abnormalities would progress during the course of the disease, which was greater in patients with a history of HE, and persisted several months after liver transplantation[23]. Currently, voxel-based diffusion tensor imaging (DTI)[27] or tract-based spatial statistics (TBSS) analysis[6] was combined to further assess the microstructural integrity alteration of the white matter (Figure 2).

As iron is a cofactor of enzymes participating in metabolism of some neurotransmitters, iron plays a vital role in brain metabolism. Abnormal deposition of iron in some specific brain regions correlates with the severity of neurocognitive impairments in patients with Alzheimer's disease and multiple sclerosis by using susceptibility weighted imaging (SWI) or T2*WI MR sequence[28,29]. T2*WI MR imaging can detect subtle iron deposition changes in the brain[29]. Liu *et al*[30] found that MHE was associated with abnormal iron deposition in the frontal-basal ganglia-thalamocortical circuits by measuring the phase value derived from corrected phase image (CPI). Their research demonstrated that there was significantly decreased phase value in the frontal cortical-

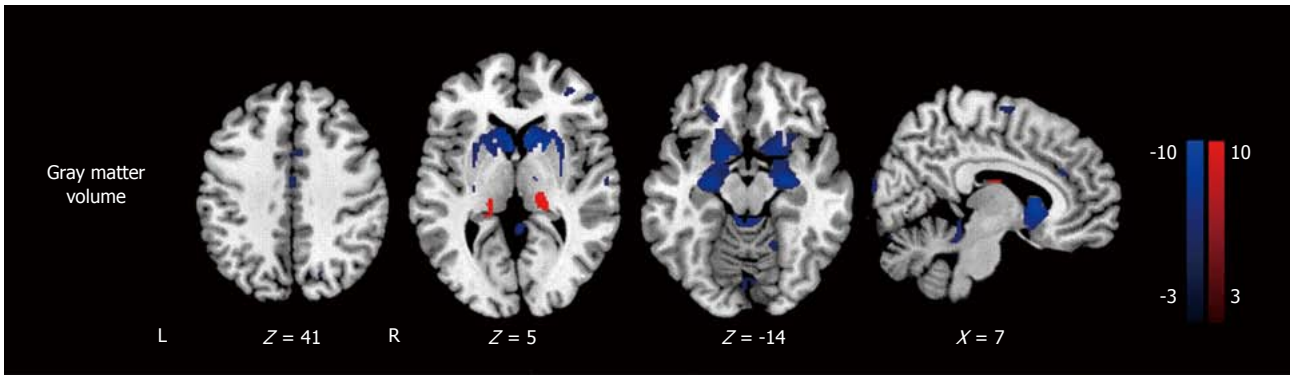

Figure 2 Voxel based morphometry analysis of patients with minimal hepatic encephalopathy and healthy controls. Minimal hepatic encephalopathy patients show grey matter volume losses in the frontal and temporal cortices, caudate, putamen, amygdale, paracentral lobule, anterior and middle cingulate cortices, supplementary motor area, and increased volume in the thalamus. From reference [6] (with permission). L: Left; R: Right.

basal ganglia circuits of MHE patients, which correlated with neurocognitive impairments by using T2*-weighted gradient echo imaging. This study may point to the potential role of iron in the pathophysiological mechanism of HE. SWI can more sensitively detect iron than T2*WI, however, no study has been reported in HE, to the best of our knowledge.

DWI AND DTI

DWI can be used to quantitatively assess the mobility of water molecules in human body. DWI has been widely employed in the central nervous system since 1990s[31]. The diffusion of water molecules abides by the principles of Brownian motion. Under an unconstrained circumstance, taking cerebrospinal fluid as an example, the movement of water molecules is random and thus equal in all directions (isotropic); while in the context of restricted environments, such as axonal myelin sheaths, water molecules tend to move parallel to, rather than perpendicular to, the white matter tracts[32]. Therefore, the motion is restricted in some directions (anisotropic). DWI can detect this subtle alteration in water molecule movement. On the basis of differently weighted diffusion-weighted images, a measure of diffusion can be calculated; ADC images can be derived from. The ADC represents tissue water diffusivity and is impacted by interactions between water molecules and their surrounding environment.

DTI can be established after the acquisition of diffusion data in a minimum of six non-collinear directions. DTI can provide detailed information on the microstructure within an imaging voxel, including fractional anisotropy and mean diffusivity[33], which can be applied to evaluate white matter integrity in different brain regions. In addition, with the help of advanced fiber tracking algorithms, 3D visualization of neural tracts can be generated to allow a direct view of white matter connectivity. Many researchers have indicated that both cytotoxic edema and interstitial edema coexist varying in degree or proportion according to the onset of HE, that is, cytotoxic edema take a predominant role in acute HE, whereas interstitial edema is more overt in chronic one. Furthermore, DWI and DTI can be used in assessing the effectiveness after manitol infusion therapy and in monitoring HE after transjugular intrahepatic portosystemic shunt (TIPS)[34,35]. Most previous DTI studies applied the region of interest (ROI) based analysis in assessing brain changes of HE, however, for a diffuse disease like HE, whole brain analysis seems to be more ideal. Qi *et al*[6] employed a novel tool, TBSS based DTI, for investigating the white matter of HE patients, which had a minimum of the registration error and personal evaluation bias.

MR SPECTROSCOPY

The most commonly used isotopes for MR spectroscopy (MRS) study in HE are ^{1}H and ^{31}P. ^{31}P MRS can detect phosphomonoesters, inorganic phosphate, phosphodiesters, phosphocreatine, γNTP, αNTP and βNTP resonances. Although these biomarkers reflect cell membrane synthesis and degradation pathways and energy metabolism information in the brain, little research consensus has been achieved owning to relatively rare ^{31}P MRS studies and more complicate application of MR technique compared with widely used ^{1}H MRS in HE study.

As a well-known noninvasive technique, ^{1}H MRS can be utilized to provide information on brain metabolites such as choline (Cho), creatine (Cr), N-acetyl aspartate (NAA), glutamine and glutamate (Glx), as well as osmolytes such as myoinositol (mIns) and taurine. Currently, there is a consensus on characteristic triad of ^{1}H MRS findings in HE (*e.g.*, intracellular Cho and mIns depletion as well as Glx accumulation), which correlates with neuropsychiatric impairment in HE patients. ^{1}H MRS findings supported the astrocyte swelling hypothesis. In brief, hyperammonia accounts for the significant elevation of astrocyte intracellular glutamine, which triggers organic osmolyte depletion (lower Cho/Cr and mIns/Cr) in patients with cirrhosis, and the increase

of glutamate and glutamine signals, to compensate for glial glutamine accumulation (higher Glx/Cr) in order to maintain osmotic homeostasis. However, astrocyte swelling may occur as a consequence of decompensation of this volume-regulatory mechanisms followed by neural-glial disturbance.

Among these ^{1}H-MRS variables, mIns seems to be a more sensitive biomarker in the early detection of HE[36,37]. In a study by Mardini *et al*[38], they administered an oral amino acid mixture to resemble haemoglobin caused by gastrointestinal bleeding as a challenge to induce hyperammonaemia. Notably, ^{1}H MRS findings exhibited a significant reduction in mIns concentration after the challenge, whereas no statistically significant difference was found in Cho, Glx and NAA. The rapid fall of mIns correlated with a compensatory mechanism to counteract glutamine accumulation. Thus, mIns alteration may be the fastest reaction to a subtle change of astrocyte water content. Cho is a component of phosphocholine and glycerophosphorylcholine, which are related to cell membrane activation. Cho is an important component for the synthesis of acetylcholine, which may be associated with memory, recognition and emotions as an important neurotransmitter. Cho/Cr reduction may underlie the cognition impairment in HE[37].

Most MRS studies found no significant changes of NAA in patients with cirrhosis, which may indicate no significant impairment of neurons during the progression of HE. It was considered previously that neuronal changes in HE was absent or unimportant in explaining the alteration of neuropsychiatric status. However, there is neuropathological evidence for significant neuronal cell loss in the brain of HE patients. For instance, dopaminergic and serotoninergic neuronal systems as well as Purkinje cells are proved to lose in HE in postmortem studies[39]. Some VBM and cortical surface-based analysis studies indirectly support the insult of the grey matter in HE patients[23,26].

Clinically, ^{1}H-MRS has been employed as a useful tool to monitor the metabolite reversibility after successful liver transplantation and effective treatment accompanied by improvement of neurologic manifestations[40]. However, the recovery of various metabolites follows different time courses. One recent study found that there was no change of Glx/Cr, Cho/Cr, mIns/Cr, or NAA/Cr ratios in pre- and post-oral L-ornithine L-aspartate therapy[41].

The current limitations of widely used one-dimensional ^{1}H-MRS in HE had two aspects, namely, spectral overlap of various metabolites resonating within a narrow spectral range, such as glutamine and glutamate, and invisibility of low concentration metabolites. Some novel MRS techniques appear to be able to address above-mentioned issues to some extent. Two-dimensional ^{1}H-MRS finding was consistent with typical findings by one-dimensional ^{1}H-MRS in HE patients with enhanced spectral resolution which can not only distinguish the spectral overlap metabolites, but also detect J-coupled metabolites, such as aspartate, taurine, and gamma-aminobutyric acid[36]. Moreover, the utility of higher magnetic field strength systems (3T or higher) can increase the spectral resolution with a shortened acquisition time.

MR PERFUSION IMAGING

It is well known that cerebral blood flow (CBF) changes in HE patients, and the CBF is redistributed from cortical areas to the basal ganglia structures, causing a heterogeneous distribution of CBF, which is established mainly by PET or single-photon emission computed tomography studies[42]. Decrease of CBF in diffuse cortical areas and the elevation of CBF in bilateral basal ganglia accounted for the accumulation of manganese, which shows high signal intensity on T1 weighted images[42]. Moreover, CBF increase in basal ganglia is correlated with clinical signs of HE[43]. Some MR perfusion imaging techniques can be used to quantitatively detect CBF. Li *et al*[44] investigated hemodynamic changes in brain basal ganglia in patients with MHE using dynamic susceptibility contrast (DSC)-enhanced MR perfusion imaging in 12 MHE patients and 10 healthy controls. Increased CBF in the basal ganglia and thalamus were found in patients with MHE.

Compared with DSC-MR perfusion imaging, arterial spin-labeling (ASL) MR perfusion imaging appears to be a more rational choice in detecting the CBF changes in HE patients due to the absence of potentially harmful MR contrast agent. In a study by Zheng *et al*[45], they assessed CBF changes in cirrhotic patients after TIPS by using ASL MRI in which a "bolus" of tagged blood was employed as an endogenous contrast agent. They exhibited that 7 out of 9 patients experienced a global CBF increase, while the remaining 2 had a global CBF decrease by 16% and 31%, respectively, who suffered from multiple episodes of OHE during follow-up. In another study, Zheng *et al*[46] further studied short- and long-term effects of TIPS on CBF in patients with cirrhosis. In this longitudinal study, the baseline CBF level was established by performing ASL MRI 1-9 d before TIPS, and the follow-up MR examinations were performed about 1 wk, 3 mo, 6-9 mo and 12-18 mo after TIPS. They found that CBF measured at different time points after TIPS insertion showed different patterns, and a sharp decline of relative CBF in the 1 wk to 3 mo period after TIPS insertion indicated that a high event rate of HE might relate with the unadaptable CBF in patients undergoing TIPS insertion. Moreover, Zheng *et al*[47] investigated the CBF changes in MHE patients, non-HE and healthy controls by using ASL MR perfusion imaging, and found that CBF of the right putamen was of the highest sensitivity (93.8%) and moderate specificity (75.0%) for characterization of MHE when using the cutoff value of 50.57 mL/min per 100 g, which indicated that CBF measured by ASL MRI can be a useful imaging marker for differentiating MHE from non-HE patients.

BLOOD OXYGENATION LEVEL DEPENDENT FUNCTIONAL MRI (BOLD fMRI)

BOLD fMRI is increasingly used in HE studies in recent years. BOLD signal is derived from the intrinsic intravascular susceptibility contrast agent deoxyhemoglobin, and is correlated with the proportion of deoxyhemoglobin/oxyhemoglobin and CBF. BOLD fMRI has supplanted dynamic susceptibility contrast imaging as the most prevalent method of fMRI for most cognitive neuroscience studies since 20 years ago[48]. Nowadays, BOLD fMRI has many utilities in various clinical fields, for instance, presurgical planning, treatment evaluation, clinical assessment, and psychiatric diagnosis[48]. Nevertheless, BOLD fMRI has been playing a vital role in cognitive neuroscience. Herein, we summary the research progress of BOLD fMRI in HE studies in recent years according to task-related fMRI and resting-state fMRI.

Task-related fMRI

As far as we know, only a few studies used task-related fMRI to investigate the neural basis of cognitive dysfunciton in HE patients. Zafiris *et al*[49] first studied neural mechanism underlying impaired visual judgment in HE patients by using task-related fMRI. They investigated 9 cirrhosis subjects without OHE and 10 healthy controls as well by using fMRI with CFF as the task. Compared to healthy controls, visual judgment-related BOLD activation was decreased in the right inferior parietal cortex (IPL) in cirrhosis patients. Moreover, the subjects exhibited impaired neural interaction between IPL and the parietooccipital cortex, the intraparietal sulcus, the anterior cingulate cortex (ACC), the right prefrontal cortex (PFC), the medial temporal lobe, and the extrastriate cortex V5. In particular, an enhanced coupling between IPL and the postcentral cortex was claimed. This study pointed to the existence of an early impaired and compensatory neural mechanism during visual judgment in cirrhosis patients without OHE. Zhang *et al*[8] carried out another study on neural mechanism of cognitive control impairment in cirrhosis by using block-designed fMRI paradigm with a modified Stroop task using Chinese characters. A cohort of 14 cirrhosis patient without OHE and 14 healthy controls were recruited in this study. There were two tasks involved in different conflict levels. Subjects were allowed to practice incongruous word-naming task (easier task) before the MRI was performed, while they were blinded to the content of incongruous color-naming task (harder task). They concluded that the impairment of the ACC-PFC-parietal lobe-TFG circuit was the neural mechanism underlying cognitive control impairment in cirrhotic patients. Most recently, Liao *et al*[50] explored the neural basis of spatial working memory impairment in MHE patients using n-back task related BOLD-fMRI. They found a neural network activation in bilateral PFC, bilateral premotor area, supplementary motor area and bilateral parietal areas, which may explain the neural basis of spatial working memory impairment[50]. McPhail *et al*[41] performed a block design task-related fMRI aiming at measuring neural activation during treatment (oral *L*-ornithine *L*-aspartate) of MHE in a longitudinal study. Their study found that posterior cingulate, ventral medial PFC and visual cortex showed increased function after successful HE treatment.

Resting-state fMRI

Currently, investigators have shifted their attention to brain activities in the resting state in HE patients. Resting-state functional connectivity has revealed a number of networks (resting-state networks) which are consistently found in healthy subjects and represent specific patterns of synchronous activity. These networks persist during task performance and sleep and under sedation. Using both ROI based analysis and independent component analysis (ICA), a number of resting-state networks are found: the default mode network (DMN), the sensorimotor component, the executive control component, up to three visual components, two lateralized frontoparietal components, the auditory component and the temporo-parietal component[51]. These resting-state networks consist of anatomically separated, but functionally connected regions displaying a high level of correlated BOLD signal activity.

Nowadays the most studied network is the DMN, including the medial PFC, rostral anterior cingulate, posterior cingulate, and precuneus. DMN is known to have high metabolic activity during rest and is relatively de-activated during cognitively demanding tasks, such as visual and auditory attention, language processing, memory, and motoric activity. Zhang *et al*[52] used the reverse subtraction method to investigate the task-related deactivation to observe the DMN in patients with hepatic cirrhosis. They found that an abnormal deactivation mode may exist in hepatic cirrhosis patients. After this, with the awareness of the potential of uncovering the pathophysiological mechanisms underlying HE by studying DMN, a number of studies have been conducted by Zhang *et al*[53] and other groups. In a standard resting-state fMRI, that is, the participants are required to rest with their eyes closed and keep their heads still and not to think of anything in particular during MR imaging scans lasting typically a few minutes, Zhang *et al*[53] employed ICA method to retrieve DMN components in the patients with HE, and found significantly reduced functional connectivity in the right middle frontal gyrus, left precuneus, and left posterior cingulate cortex (PCC) in the patients with HE. *Z* scores of the left angular gyrus and left PCC were found to have a negative correlation with venous blood ammonia levels in the HE group.

Accumulating resting-state fMRI evidence suggests that an alteration of cortico-striato-thalamic pathway may play an important role in HE. Zhang *et al*[54] studied the patterns of whole-brain functional connectivity in patients with MHE by defining connectivity of interest

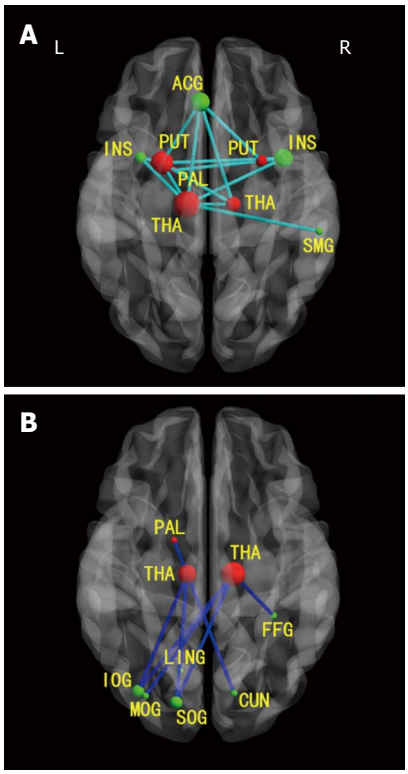

Figure 3 Axial magnetic resonance images show functional connectivities in patients with minimal hepatic encephalopathy between cortical and subcortical regions. A: Decreased positive functional connectivities between cortical and subcortical regions; B: Decreased negative functional connectivities between cortical and subcortical regions. Green nodes: Cortical ROIs; red nodes: Subcortical ROIs; light blue lines: Decreased positive connectivities in patients with minimal HE; dark blue lines: Decreased negative connectivities in patients with minimal HE. R: Right; L: Left; ACG: Anterior cingulum gyrus; SMG: Supramarginal gyrus; PUT: Putamen; PAL: Pallidum; THA: Thalamus; CUN: Cuneus; LING: Lingual gyrus; SOG: Superior occipital gyrus; MOG: Middle occipital gyrus; IOG: Inferior occipital gyrus; FFG: Fusiform gyrus; INS: Insula. From reference [54] (with permission).

(COI) as significantly changed connectivity of every two ROIs compared with controls for further analysis (Figure 3). All 22 COIs related to subcortical ROIs (bilateral putamen, pallidum, and thalamus) were weaker in patients with MHE. Of 29 cortical COIs, 22 connectivities were weaker and 7 were stronger in patients with MHE. In addition, nearly all COIs with significant differences correlated with neuropsychological impairment. In particular, impairment in the basal ganglia-thalamocortical circuit could play an important role in mediating neurocognitive dysfunction, especially for psychomotor speed and attention deficits in patients with MHE. Additionally, some studies used ROI method to investigate the functional connectivity of the above-mentioned circuit in HE patients and further demonstrated the abnormal circuit in HE[55-57]. For example, Qi *et al*[58] found that MHE patients had disrupted thalamic functional connectivity which indicated reduced integrity of thalamic resting state network in MHE by using an ROI-based method.

Rather than above-mentioned studies on brain network functional connectivity, a "small-world" network model can quantify the effectiveness of information transfer among widely distributed brain regions. The human brain is organized intrinsically as highly modular small-world architectures. In this model, the information transfer is highly efficient, which has been attributed to the brain's network organization. Hsu *et al*[59] applied small-world topology to assess the alteration of functional connectivity in HE patients. They found that HE patients showed abnormal small-world properties, which were related to HE grade. The balance between local specialization and global integration of brain functional activity was disrupted in HE patients.

Resting-state fMRI can be used to monitor or predict progression of HE. Qi *et al*[60] demonstrated that patients with HE had diffuse abnormalities in intrinsic brain activity with amplitude of low frequency fluctuations (ALFF), based on whole-brain functional analysis algorithms (Figure 4). The levels of decreased ALFF in the DMN and increased ALFF in the posterior insular cortex are dependent on the severity of HE, suggesting continuous impairment of the DMN and a compensatory role of the insula during the progression of HE. Chen *et al*[9] studied resting-state functional connectivity within DMN in patients with MHE and a history of OHE, and found that previous OHE rather than current MHE might be primarily related to brain dysfunction in patients with latent OHE.

Resting-state fMRI was also used to investigate dynamic changes of brain function following TIPS and it can predict the development of HE following TIPS. ALFF can be applied as a marker in monitoring dynamic changes of intrinsic brain activity in cirrhotic patients after TIPS[61]. Hence, resting-state fMRI with ALFF analysis may be a noninvasive modality to detect the progression of HE, which was reported by Chen *et al*[62] in one investigation of low-grade HE.

MULTIMODALITY MRI

fMRI is a promising tool in studying HE, and it can have a flourishing prospect in near future. However, the combination of fMRI and other MR techniques, such as DTI, can provide some new insights into the understanding of pathological mechanism of brain function changes in patients with HE[63-65]. Investigators combined DTI and resting-state fMRI to investigate brain changes in MHE in a single institution study[63] and found that MHE patients have both disturbed structural and functional connectivities within the DMN. The decreased functional connectivity was also detected between some regions without abnormal structural connectivity, suggesting that the former may be more sensitive in detecting the early abnormalities of MHE. This study does not support the idea that cerebral edema play a major role in HE, and, thus, extends our understanding of the pathophysiology of MHE. With the help of fMRI and DTI as well, Lin *et al*[64] found that reduction of functional connectivity of DMN is heavier in patients with overt HE

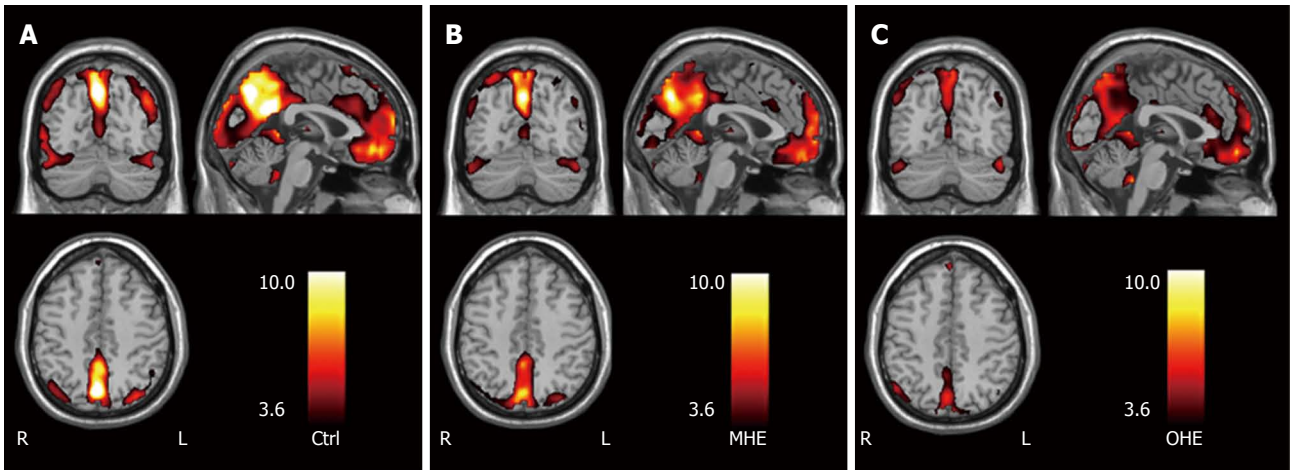

Figure 4 Amplitude of low frequency fluctuation maps in groups of healthy control subjects, patients with minimal hepatic encephalopathy, and patients with overt hepatic encephalopathy. Group of MHE and group of OHE *vs* control subjects, $P < 0.05$. Within each group (A: Group of Ctrl; B: Group of MHE; C: Group of OHE), posterior cingulated cortex and precuneus, medial prefrontal cortex, inferior parietal lobe, and occipital areas show high amplitude of low frequency fluctuation values. Color scale indicates *t* values. From reference [60] (with permission). Ctrl: Control subjects; MHE: Minimal hepatic encephalopathy; OHE: Overt hepatic encephalopathy. L: Left; R: Right.

Table 1 Imaging techniques applied in the diagnosis and investigation of hepatic encephalopathy

MR technique	MR findings or applications
Structural MRI	Diffuse cortical overt brain edema on T2WI and fluid attenuated inversion recovery in acute HE; Bilateral basal ganglia high signal intensity on T_1WI in chronic HE; Regional gray matter volume reduction, increased thalamus volume and white matter abnormality
T_2* weighted imaging	Abnormal iron deposition in the frontal-basal ganglia-thalamocortical circuits
Diffusion weighted imaging	High signal intensity in subcortical areas and low apparent diffusion coefficient in acute HE; High apparent diffusion coefficient in chronic HE
Diffusion tensor imaging	Increased mean diffusivity and decreased fractional anisotropy in chronic HE
^{1}H MR spectroscopy	Depletion of choline and myoinositol; Accumulation of glutamine/glutamate
Dynamic susceptibility contrast-enhanced MR perfusion imaging[1]	Increased cerebral blood flow in the basal ganglia and thalamus
Arterial spin-labeling MR perfusion imaging[1]	Increased cerebral blood flow; To early diagnose HE or predict overt HE after transjugular intrahepatic porto-systemic shunt
Task-related functional MRI[1]	Attention, visual judgment and working memory impairment in HE
Resting-state functional MRI[1]	Functional connection alteration of cortico-striato-thalamic pathway; Abnormal small-world properties; Resting state network (especially default mode network) abnormalities: reduced functional connectivity in the right middle frontal gyrus, left precuneus, and left posterior cingulate cortex in the patients with HE

[1]Techniques mainly for research, currently. MRI: Magnetic resonance imaging; HE: Hepatic encephalopathy.

than in those without HE and MHE. One study by Chen *et al*[65] also indicated that both functional and structural impairments were evident after apparent recovery from OHE, suggesting that brain dysfunction induced by HE persisted after clinical resolution by combining both VBM and ROI-based fMRI.

The MR imaging findings and potential applications of these advanced MR techniques in brain changes of cirrhotic patients are summarized in Table 1.

CONCLUSION

In summary, neuropsychiatric abnormalities of HE are from a combination of multiple synergistic precipitating factors. Multimodality MR imaging is an effective and feasible research tool to uncover the pathophysiological mechanism of HE, and it will play an increasingly important role in the early diagnosis, prognosis and monitoring of HE. In particular, the combination of fMRI and other advanced MR modalities such as DTI should be widely applied in this field to deepen our understanding of the whole story of HE.

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P- Reviewer: Felipo V, Hashimoto N, Maruyama H, Trifan A
S- Editor: Ma YJ **L- Editor**: Wang TQ **E- Editor**: Zhang DN

World Journal of Gastroenterology

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DOI: 10.3748/wjg.v20.i32.11273

World J Gastroenterol 2014 August 28; 20(32): 11273-11280
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Enteric glial cells and their role in the intestinal epithelial barrier

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Supported by the National Natural Science Foundation of China, NSFC, No. 81200270; and the Scientific Research Foundation for Outstanding Young Scientist of Shandong Province, No. BS2012SW012
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Received: January 12, 2014 Revised: March 11, 2014
Accepted: May 12, 2014
Published online: August 28, 2014

Abstract

The intestinal epithelium constitutes a physical and functional barrier between the external environment and the host organism. It is formed by a continuous monolayer of intestinal epithelial cells maintained together by intercellular junctional complex, limiting access of pathogens, toxins and xenobiotics to host tissues. Once this barrier integrity is disrupted, inflammatory disorders and tissue injury are initiated and perpetuated. Beneath the intestinal epithelial cells lies a population of astrocyte-like cells that are known as enteric glia. The morphological characteristics and expression markers of these enteric glia cells were identical to the astrocytes of the central nervous system. In the past few years, enteric glia have been demonstrated to have a trophic and supporting relationship with intestinal epithelial cells. Enteric glia lesions and/or functional defects can be involved in the barrier dysfunction. Besides, factors secreted by enteric glia are important for the regulation of gut barrier function. Moreover, enteric glia have an important impact on epithelial cell transcriptome and induce a shift in epithelial cell phenotype towards increased cell adhesion and cell differentiation. Enteric glia can also preserve epithelial barrier against intestinal bacteria insult. In this review, we will describe the current body of evidence supporting functional roles of enteric glia on intestinal barrier.

Key words: Enteric glia cells; Intestinal epithelial cells; Intestinal barrier function; Tight junctions

Core tip: This review offers a state-of-the-art discussion on the role of enteric glial cells (EGCs), an intriguing population of astrocyte-like cells within the gastrointestinal tract, on the regulation of intestinal epithelial barrier. The discussion will shed light on the novel mechanisms of EGC-intestinal epithelial cells interactions, which is invaluable in ultimately developing new therapeutic tools for the restoration of the intestinal barrier functions.

Yu YB, Li YQ. Enteric glial cells and their role in the intestinal epithelial barrier. *World J Gastroenterol* 2014; 20(32): 11273-11280 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11273.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11273

INTRODUCTION

The intestinal epithelial barrier (IEB) serves as the first boundary of defense between the organism and the luminal environment. It consists of a continuous monolayer of proliferating and differentiating intestinal epithelial cells (IECs) maintained together by intercellular junctional complex which establishes the cellular polarity and reduces the space between adjacent cells. Therefore, the IEB provides a highly selective permeability that prevents the passage of pathogens[1,2]. Loss of this barrier integrity would allow the translocation of the normally excluded

luminal contents (microbes, food antigens, *etc.*) into the mucosa, where inflammatory disorders and tissue injury are initiated and perpetuated[3-5]. Considerable evidence indicates that intestinal barrier dysfunction plays a pathogenic role in diseases such as inflammatory bowel disease, celiac disease and irritable bowel syndrome[6-8]. Therefore, mediators associated with reinforcing or re-establishing IEB functions could be of great interest in the intervention of these barrier dysfunction diseases.

Beneath the intestinal epithelial cells lies a population of astrocyte-like cells that are known as enteric glia cells (EGCs). The morphological characteristics and expression markers [glial fibrillary acidic protein (GFAP), S-100β] of these enteric glia cells were identical to the astrocytes of the central nervous system[9,10]. Within the central nervous system, the blood-brain barrier, which shelters the nervous system from circulating blood, is maintained *via* interactions between astrocytes and cerebral endothelia[11]. Whether similar interactions between enteric glia and epithelia regulate intestinal barrier function has moved into the spotlight in recent years. In this review, we will summarize the current evidence supporting functional roles of enteric glia in the control of IEB functions and gut homeostasis.

INTESTINAL EPITHELIAL BARRIER STRUCTURE

The important component of the intestinal barrier is the intercellular junctional complex, which consists of the tight junctions (TJs), gap junctions, adherens junctions and desmosomes[12-14]. TJs seal the space between adjacent epithelial cells near the apical surface. Structurally, the TJs are composed of membrane-spanning proteins, including claudins, occludin, and zona occludens[15,16]. Claudins, in particular, play a critical role in barrier function. Claudin-1, claudin-4 and claudin-5 reduce paracellular diffusion by sealing neighbor epithelial cells. Conversely, claudin-2 forms channels or pores contributing to epithelial leakiness[17,18]. Occludin, and the zona occludens could link the cytoplasmic component of the TJs to the actin-myosin cytoskeleton[19,20]. Adherens junctions are located beneath the TJs and are involved in cell-cell adhesion and intracellular communication[21]. As for gap junctions and desmosomes, they are reported to be involved in cell-cell adhesion and intracellular communication, respectively[22,23]. The intercellular junctional complex forms a selective barrier which allows nutrient absorption and defends against entry of infectious agents and foreign antigens into the body. Candidate mediators are reported to affect the intercellular junctional complex in two ways, first by expression regulation[24] and second, perhaps more importantly, by affecting the redistribution processes[25]. Further, the balance between apoptosis and regeneration of epithelial cells is also crucial for the maintenance of the intact mucosal barrier function[26]. Both disruption of the intercellular junctional complex and abnormal epithelial cell apoptosis have been reported to be involved in the development of a "leaky" gut, which may promote the translocation of luminal antigens into the colonic mucosa and subsequently destroy gut mucosal homeostasis.

ENTERIC GLIA IN THE INTESTINAL TRACT

The first description of EGCs within the gut was made in 1899[27]. However, for decades, the role of EGCs in gut was largely ignored, and was considered merely as foster cells accompanying and supporting enteric neurons. Interestingly, the current body of evidence expands the functional role of these cells within the gut.

In the intestine, the EGCs are the major constituent of the enteric nervous system and outnumber enteric neurons by a factor of 4 to 10[28]. They possess a densely integrated array of intermediate filaments rich in GFAP[29] and express the calcium-binding protein S-100β[30]. The mucosal EGC population are in close proximity (< 1 μm) to the epithelial cells of the colonic crypts and their terminal end-feet processes often extend to the epithelial basement membrane and blood capillaries in the intestinal mucosa[10,28]. Meanwhile, major populations of enteric glia are found in enteric ganglia in the submucosal and myenteric plexuses of the enteric nervous system. These EGCs can ensheath the neuronal cell bodies within the enteric ganglia, as well as the connecting enteric neuronal interganglionic processes and the processes extending from the enteric plexi to the muscularis mucosae and externae, blood vessels, and mucosal glands[10,31]. The EGCs are typically described as highly irregular, stellate-shaped, small cells which provide regulatory signals for the development and function of neurons and ganglions in the gastrointestinal tract[32,33]. As is known, enteric glia share many structural and functional similarities to astrocytes in central nervous system. Amounts of evidence indicate the critical role of astrocytes in the maintenance of the blood-brain barrier. As for EGCs, it has been demonstrated that EGCs actively receive and propagate signals, both to and from nearby enteric neurons and the intestinal epithelium[27,34]. Thus, EGCs may be an ideal candidate cell type to maintain proper intestinal epithelial barrier integrity (Figure 1).

ENTERIC GLIA AND BARRIER FUNCTION

Several lines of evidence implicate an essential role of mucosal EGCs for the integrity of the gut epithelium. Examination of noninvolved intestinal tissue from patients with Crohn's disease demonstrated that the EGC network was significantly disrupted, and the diminished EGC network appeared to respond poorly to inflammatory signals in these patients[7,35]. Animal studies demonstrated that the conditional genetic ablation of EGC in mice could induce the disruption of the intestinal integrity and vascular disturbances, and ultimately lead to fatal hemorrhagic jejuno-ileitis[6,36,37]. Further observations showed that the destruction of the EGC network by chemical or autoim-

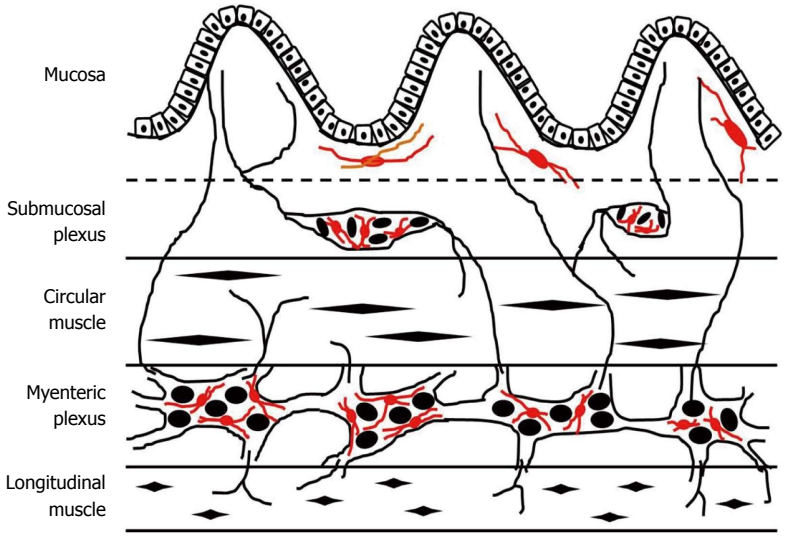

Figure 1 General distribution of enteric glia in the gut wall. The glia cells are represented in red. Mucosal glia lie in the mucosa directly beneath the intestinal epithelial cells. Major populations of enteric glia are found in enteric ganglia in the submucosal and myenteric plexuses of the enteric nervous system.

mune T-cell-targeted methods resulted in a collapse of the epithelial lining, and the mucosa healing was obviously delayed[7,38,39]. In a mice model of intestinal injury caused by severe burns, stimulation of the vagus nerve could activate the EGCs, and the activated EGCs subsequently prevented burn-induced intestinal permeability and attenuated histological gut injury[40]. Hence, it is imaginable that EGCs are a major constituent of the IEB microenvironment favoring barrier protection.

EGCS MEDIATORS AND MUCOSAL BARRIER FUNCTION

Mucosal EGCs lie in the mucosa directly beneath the epithelial cells, suggesting that regulation of IEB functions by EGC might be *via* paracrine pathways[41]. As is known, mucosal EGCs are producers of several mediators implicated in mucosal barrier function[42], such as glial-derived neurotrophic factor (GDNF), transforming growth factor-β1 (TGF-β1), 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (15dPGJ2), glial-derived s-nitrosoglutathione (GSNO) and neurotrophins.

GDNF

GDNF potently promotes the survival and differentiation of many types of neurons[43,44], and is able to prevent apoptosis of neurons induced by axotomy[45]. In the intestine, the main source of GDNF could be identified as the EGCs of the mucosal plexus. It is reported that GDNF immunoreactivity was strongly up-regulated in the colonic epithelium during rat experimental colitis and GDNF had strong anti-apoptotic effects on the colonic epithelial cells[46,47]. Mechanism studies revealed that the GDNF-mediated antiapoptotic properties required the activation of both the MAPK and the PI3K/AKt signaling pathways[46,47]. Recent evidence further supports the anti-apoptotic role of GDNF on mucosal EGCs. It showed that GDNF could feed back in an autocrine manner to protect EGCs from apoptosis in Crohn's disease patients[48]. Disruption of this protective network could contribute to a higher susceptibility towards EGC apoptosis, and subsequently induce alteration of the mucosal integrity. Abnormal mucosal immune responses are considered as a contributing event in the mucosal barrier dysfunction. At this point, role of GDNF in the mucosal inflammatory responses was also investigated. Interestingly, GDNF could inhibit the expression of pro-inflammatory cytokines [interleukin (IL)-1β, tumor necrosis factor (TNF)-α] and myeloperoxidase activity in the rat colon[47,49]. In addition, administration of the recombinant adenoviral vectors encoding GDNF (Ad-GDNF) *via* the rectum could significantly ameliorate the severity of dextran sodium sulfate-induced rat colitis[47].

TGF-β1

TGF-β1 has been reported to be secreted by astrocytes and plays a key role in neuronal homeostasis[50]. Interestingly, phenotypic studies of EGCs revealed that TGF-β1 could also be synthesized and released by EGCs[28,51]. Moreover, TGF-β1 was reported to account for approximately 12%-30% of the effects of EGCs on intestinal epithelial cell proliferation[52,53]. Growing evidence demonstrates that TGF-β1 inhibits epithelial cell proliferation while stimulates epithelial cell migration in a dose-dependent manner[54,55]. TGF-β1 mediates the anti-proliferative effects through either a down-regulation of cyclin-dependent kinases or an up-regulation of cyclin-dependent kinase inhibitors, and consequently induces a cell cycle blockade in the G_1/S phase[56,57]. Interestingly, the effect assessment of cultured EGCs on cultured epithelial cell lines supports the concept that EGCs could significantly inhibit intestinal epithelial cell proliferation and concomitantly increase the cell surface of epithelial cells partly through a TGF-β1-dependent pathway[28].

15dPGJ2

15dPGJ2, a cellular source of the natural peroxisome proliferator-activated receptor gamma (PPARγ) ligand, could also be provided by EGCs[58]. Through activation of PPARγ, 15dPGJ2 mediated the inhibitory effects of EGC on epithelial cells proliferation and the positive effects of EGCs on epithelial differentiation, which promises the continuous renewal process of the intestinal epithelium[59]. As is known, the renewal process of IECs involves the epithelial cell emergence from the mucosal crypts and subsequent cell migration along the crypt-villus axis, during which the IECs cease to proliferate and acquire differentiated function. However, it should be noteworthy that EGC-derived 15dPGJ2 had no effect on the colonic paracellular permeability[60]. Further evidence showed that the anti-proliferative effects of EGCs might be attributed to its induction of a cell cycle blockade at G0/G1 phase in epithelial cells[61,62]. Besides, Krüppel-like factor 4, which is expressed in IECs and plays major roles in IEC differentiation and maturation, was supposed to be the candidate cellular target of PPARγ following activation by EGCs[63].

GSNO

GSNO is another potent barrier-inducing factor present in enteric glial cell-conditioned media. Interestingly, GSNO is the nitrosylated form of reduced glutathione (GSH), and nitrosylation of GSH is responsible for an antioxidant cytoprotective action[64]. It has been demonstrated that intraperitoneal administration of GSNO obviously inhibited the increased intestinal permeability induced by enteric glial cell ablation in transgenic mice. This barrier-inducing effect of GSNO might be associated with the up-regulated expression of peri-junctional F-actin and TJ-associated proteins such as zonula occludens-1 (ZO-1) and occludin[65]. GSNO may also maintain the epithelial barrier function by improving the localization of the intestinal tight junction proteins, such as ZO-1, occudin and phosphorylated MLC[66]. In addition, GSNO may inhibit the gut inflammatory response through redox-sensitive s-nitrosylation of nuclear factor κB (NF-κB) inflammatory signaling, suppressing the transcription of pro-inflammatory mediators such as TNF-α[67]. Altering NF-κB inflammatory signaling also has important effects on the down-regulation of endothelial cell adhesion molecules that promote leukocyte infiltration[68]. However, it should be noteworthy that GSNO did not regulate the epithelial barrier in a dose-dependent manner. It was reported that disruptive effect of GSNO on the epithelial integrity was obtained at relatively higher concentrations[69]. The molecular mechanism remains unclear, but may be attributed to altered NO production. As is known, GSNO is a potent nitric oxide donor, which can function to *S*-nitrosylate proteins and play an important role in proper epithelial ion transport[70,71].

ROLE OF EGC ON IEC FUNCTIONS

In concert with the barrier-inducing effects of EGCs, microarray analysis was carried out to further identify the EGC influence on the intestinal epithelial cells transcriptome. The study was performed to identify statistically significant differences in gene expression profiling in Caco-2 cells cultured alone or in presence of EGCs. The results showed that EGCs could regulate the expression of various genes involved in the control of IEC adhesion, differentiation, motility, cell cycle and proliferation. These collective gene-related data reinforces the concept that EGCs play a major protective role upon IEB homeostasis[72]. Besides the protective role, a repair process-inducing role of EGCs has recently been put forward. The study showed that EGCs could promote mucosa healing by increasing epithelial restitution and cell spreading after mechanical injury to IEC monolayers. Epidermal growth factor precursor (proEGF), as a novel glial mediator, was confirmed to be involved in the EGC-mediated epithelial restitution[73]. Indeed, proEGF exhibits a lower wound healing ability compared with EGF. However, subsequent studies showed that EGC-derived proEGF could be activated by concomitant release of MMPs or proteases during inflammatory or infectious insults of IEB, which would process proEGF into mature EGF and therefore enhance mucosa repairing[73,74].

EGC PRESERVE IEB FROM BACTERIAL INSULT

Recently, a protective role of EGCs on the mucosal barrier during enteric bacterial insult has drawn increasing interest, which may also provide new therapeutic tools in the protection and regeneration of intestinal barrier[75]. As is known, *Shigella flexneri* (*S. flexneri*) is one of the major enteroinvasive pathogens which are responsible for the destruction of the intestinal epithelium[76]. Flamant demonstrated for the first time that the protective effects of EGCs on the IEB could be due in part to its ability to inhibit *S. flexneri* invasion. Further, cdc42, a key molecular factor for *S. flexneri* invasion, was significantly down-regulated by performing co-culture experiments between IECs and EGCs. In addition, EGCs prevents tight-junction disruption during *S. flexneri* infection, and diminishes mucosal secretion of the pro-inflammatory cytokine IL-8[77]. Indeed, these EGC-mediated effects could also be reproduced by GSNO[78]. Under the stimulation of lipopolysaccharide (LPS), EGCs could play the protective effect on IEB functions by inhibiting the increase of inducible nitric oxide synthase activity induced by LPS[79]. A recent study has shown that Toll-like receptor 2 (TLR2), which plays key roles in sensing microbial structures, is expressed on glial cells. In the intestine, TLR2 exerts cytoprotective effects in intestinal epithelial cells and regulates epithelial barrier function. Besides, TLR2 could stimulate the intestinal expression of GDNF through NF-κB and p38 mitogen-activated protein kinase signaling pathway. In this context, the TLR2-GDNF axis might represent an attractive regulator for gut homeostasis[80].

EGCS AND INTESTINAL MUCOSAL INFLAMMATION

Because gut inflammation accompanies changes in intestinal permeability, roles of EGCs in mucosal inflammation have also been investigated. Similar to CNS astrocytes, EGCs are recognized as immunocompetent cells that have the ability to express major histocompatibility complex class I and class Ⅱ molecules, and to produce and respond to a variety of chemokines and cytokines[10]. Co-cultured with interferon-gamma and TNF-α, EGCs acquire the ability to process and present antigens efficiently to specific T-cells, indicating that EGCs can act as antigen-presenting cells[7]. EGCs express substance P, which can induce the activation of mast cells and macrophages and promote lymphocyte proliferation[81]. S100B protein, specifically expressed by EGCs, can orchestrate a wide range of signal activation pathways which are directly correlated with the severity of gut inflammatory processes[82,83]. Palmitoylethanolamide can exert anti-inflammatory effects through the selective targeting of the S100B/TLR4 axis on EGCs, causing a downstream inhibition of nuclear factor kappa B-dependent colonic inflammation[84]. Further, EGCs have the ability to respond to inflammatory stimuli through the production of pro-inflammatory cytokines, such as IL-6[85], TGF-β_1[28] *etc*. EGCs could also inhibit inflammation in animal models of colitis as they produce mediators such as nerve growth factor and neurotrophin-3 which have anti-inflammatory properties[86,87]. In Cytomix-stimulated intestinal epithelial cells while EGCs were removed from the culture, the anti-inflammatory effects of nicotine were lost and consequently resulted in increased *in vitro* epithelial permeability[88]. These data support the hypothesis that EGCs are likely immune mediators in the gastrointestinal tract. However, so far, limited information is available to indicate the exact mechanisms of EGCs in the regulation of mucosal inflammation-induced permeability alterations.

Collectively, EGCs, intriguing cellular populations within the gastrointestinal tract, might be of interest as a source of novel molecules aiming at preventing relapse or increasing IEB repair. However, the precise mechanism of EGCs on the regulation of intestinal barrier is still partly unclear. Future research identifying precisely how EGCs participate in intestinal epithelium physiology and pathophysiology will be beneficial for our understanding of EGC-IEC interactions, which is also invaluable in ultimately developing new therapeutic tools for the restoration of the barrier functions.

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P- Reviewer: Bashashati M **S- Editor**: Gou SX
L- Editor: O'Neill M **E- Editor**: Wang CH

World Journal of Gastroenterology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i32.11281

World J Gastroenterol 2014 August 28; 20(32): 11281-11286
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Hepatitis C in the pediatric population: Transmission, natural history, treatment and liver transplantation

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Received: January 30, 2014 Revised: April 12, 2014
Accepted: May 12, 2014
Published online: August 28, 2014

Abstract

The number of children affected by the hepatitis C virus (HCV) in the United States is estimated to be between 23000 to 46000. The projected medical cost for children with HCV in the United States is upwards of 200 million over the next decade. The implementation of routine screening of blood supply has virtually eliminated transmission *via* transfusion and vertical transmission is now the most common mode of infection in children. Infections acquired during infancy are more likely to spontaneously resolve and fibrosis of the liver tends to increase with age suggesting slow progressive histologic injury. Anti-viral treatment may be warranted in children with persistently elevated liver enzymes or with significant fibrosis on liver biopsy. Current standard of care includes weekly pegylated interferon and ribavirin twice daily. Predictors of high sustained viral response include genotype 2 and 3 and low viral load in children with genotype 1 (< 600000 IU/mL). Triple therapy is associated with a significantly higher rate of sustained virologic response (> 90%). Only 34 pediatric patients were transplanted with hepatitis C between January 2008 and April 2013. The majority of pediatric patients were born prior to universal screening of blood products and, as of June 2013, there are only two pediatric patients awaiting liver transplantation for end-stage liver disease secondary to hepatitis C. Pediatric survival rates post-transplant are excellent but graft survival is noticeably reduced compared to adults (73.73% for pediatric patients at one year compared to 87.69% in adult patients). New safe potent, and all-oral effective antiviral therapies for recurrent HCV should help increase graft survival.

Key words: Hepatitis C; Liver transplantation; Pediatric; Infection; Fibrosis; Liver disease

Core tip: The number of children affected by the hepatitis C virus (HCV) in the United States is between 23000 to 46000. Current standard of care treatment includes weekly pegylated interferon and ribavirin twice daily. New enrollment into phase 1 and 2 trials with triple therapy are currently on hold due to the upcoming availability of all oral, interferon-free, direct acting antivirals. Triple therapy is associated with a rate of sustained virologic response (> 90%). Only 34 children were transplanted with HCV between January 2008 and April 2013. Pediatric survival rates post-transplant are excellent but graft survivals are reduced compared to adults. New antiviral therapies for recurrent HCV should help increase graft survival.

Khaderi S, Shepherd R, Goss JA, Leung DH. Hepatitis C in the pediatric population: Transmission, natural history, treatment and liver transplantation. *World J Gastroenterol* 2014; 20(32): 11281-11286 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11281.htm DOI: http://dx.doi.

org/10.3748/wjg.v20.i32.11281

INTRODUCTION

Children represent a small but important portion of those infected with the hepatitis C virus (HCV) and our understanding of the disease is limited in the pediatric population. HCV differs in children with regards to transmission, rates of clearance, natural history and treatment. This review aims to summarize our understanding of the major issues related to HCV in children, including pediatric liver transplantation.

EPIDEMIOLOGY

The prevalence of hepatitis C antibody (anti-HCV Ab) in North America is estimated to be about 1% to 1.5%[1] and children represent an even smaller percentage. Recent data from the National Health and Nutrition Examinations Survey Ⅲ (NHANES Ⅲ) report that 0.17% of 6- to 11-year-olds and 0.39% of 12- to 19-year-olds are anti-HCV positive. Although the proportion of children who are anti-HCV positive who are also HCV RNA positive is unclear, the number of children affected by chronic HCV infection in the United States is estimated to be between 23000 and 46000[2].

The prevalence of HCV is higher in children who received blood products prior to 1992 when routine screening of blood supply was instituted and a second-generation ELISA test was introduced. Most of these affected individuals had conditions such as hemophilia, malignancy or congenital heart disease and the risk of chronic HCV is related to the amount of blood products received[3-5].

Hepatitis C has projected medical costs of a staggering $10.7 billion for adults in the United States in the years 2010-2019[6] and $199-336 million for children over the next decade[2]. Its impact on morbidity includes a 26-fold increased risk of liver-related death when acquired during childhood[7].

TRANSMISSION

The implementation of routine screening of the blood supply has virtually eliminated transmission *via* transfusion, and vertical transmission is now the most common mode of infection in children. There are approximately 8000 new cases of hepatitis C per year in the United States from vertical transmission[2]. The estimated risk of transmitting HCV from mom to child is 4.3% in a mother with detectable HCV RNA[8]. A maternal HCV load of 600000 IU/mL or higher increases the risk of mother-to-infant transmission[9]. A combination of HCV and HIV infection increases the risk of vertical transmission two to three fold[9]. Fortunately, the rate of HCV transmission seen in pregnant HIV/HCV coinfected women normalizes to that of mono-infected mothers when maternal HIV activity is controlled with highly active antiretroviral therapy (HAART)[10].

There are inconsistent reports on the role that mode of delivery may play in the risk of vertical transmission. Delivery by cesarean section is not routinely recommended as it provides no added benefit in reducing the risk of perinatal transmission[8,11]. However, prolonged rupture of membranes, placement of fetal scalp monitors, exposure to contaminated maternal blood and fetal anoxia at the time of delivery all have been associated with increased risk of perinatal HCV infection[11,12]. The precise timing and process by which the virus is transmitted from mother to infant are unknown but recent data suggest transmission is more likely to occur *in utero* than during the perinatal period[13].

NATURAL HISTORY

Infections acquired during infancy are more likely to spontaneously resolve than those acquired as an adult. In a large, multi-center, prospective study in Europe, 266 children with vertical HCV infection were followed for a median of 4.2 years[14]. Approximately twenty percent cleared the infection while 80% remained chronically infected. Children who remained HCV RNA PCR positive positive during and after one year of age had a lower likelihood of clearance.

Higher rates of spontaneous resolution have been found in infants with the Rs12979860 CC genotype for the IL28B polymorphism[15]. Infants, in particular, may have defense mechanisms that explain the inefficiency of HCV perinatal transmission. The placenta has been shown to play an immunoprotective role against HCV transmission in the neonate, and infants with human leukocyte antigen DR13 are also less likely to develop chronic HCV from vertical transmission.

As in adults with chronic HCV, fibrosis of the liver in pediatric patients tends to increase with age suggesting slow progressive histologic injury[16-18]. Although uncommon, progression to cirrhosis in childhood has been reported. A large multi-center Italian study analyzed 504 consecutive anti-HCV antibody positive patients over a 15 year period[19]. Nearly 95% were HCV RNA positive and the majority (56%) acquired HCV vertically. Although 8% demonstrated spontaneous clearance, 1.8% developed cirrhosis in a 2-9 year period. Risk factors for developing cirrhosis included genotype 1a and steatosis.

SCREENING

Screening for hepatitis C should be considered in those children with risk factors for HCV. The largest group is comprised of children born to HCV-infected mothers or mothers with a history of intravenous drug abuse. Other groups include children with HIV infection, children who are victims of a sexual assault, children with a history of multiple sexual partners, and adolescents with a history of intravenous drug use.

Table 1 Selected pegylated interferon/ribavirin treatment trials in children with chronic hepatitis C infection *n* (%)

Ref.	*n*	Treatment	Sustained virological response		
			All types	HCV type 1	HCV type 2/3/6
Wirth *et al*[26]	41	IFN-2b-ribavirin	25 (61)	18 (53)	7 (100)
González-Peralta *et al*[25]	118	IFN-2b-ribavirin	54 (46)	33 (36)	21 (84)
Wirth *et al*[27]	62	PEG-IFN-2b-ribavirin	36 (59)	22 (48)	13 (100)
Wirth *et al*[28]	107	PEG-IFN-2b-ribavirin	70 (65)	38 (53)	28 (93)
Schwarz *et al*[29]	55	PEG-IFN-2a-ribavirin	29 (53)	21 (47)	8 (80)
Schwarz *et al*[29]	59	PEG-IFN-2a	12 (21)	8 (17)	4 (36)

Modified from reference [20]. PEG-IFN: Pegylated interferon; HCV: Hepatitis C virus.

MONITORING

The North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition recently reviewed available data to assist providers in the diagnosis, management and prevention of HCV infection in children and adolescents[20]. Newly diagnosed pediatric patients should undergo a thorough physical exam and laboratory evaluation to determine risk factors for infection and to detect the presence of associated sequelae of liver disease. Annual alfa-fetoprotein or liver ultrasound is also recommended in the setting of elevated transaminases during non-treatment monitoring. Liver biopsy is not always necessary but should be considered if the results will influence clinical decision making (for example, patients being considered for antiviral treatment or to exclude comorbid disease such as autoimmune hepatitis). HCV-infected patients with significant fibrosis or cirrhosis should be monitored annually with alpha-fetoprotein and abdominal ultrasound[21].

CLINICAL SIGNS AND SYMPTOMS

The clinical course of acute and chronic HCV in children is generally benign. Symptoms are often non-specific and mild. Progression to decompensated liver disease may occur but is rare in children. Growth is generally unaffected in young children with chronic HCV[22] and biochemical markers of liver dysfunction will fluctuate. Transaminases may be normal or minimally elevated in chronic HCV and in some cases may remain elevated despite anti-HCV seronegativity[23,24].

TREATMENT

Determining candidacy for treatment of chronic HCV in a child or adolescent is often controversial. Annual follow-up until adulthood when superior medications will likely be available may be a good option for children and adolescents who have no indicators of progressive disease. Anti-viral treatment, however, may be warranted in children with persistently elevated liver enzymes or those with significant fibrosis on liver biopsy.

The current standard of care includes pegylated interferon once weekly in combination with ribavirin twice daily. Several smaller single-center and large multicenter pediatric studies have proven the superiority of achieving sustained virologic response (SVR), defined as being HCV RNA negative 6 mo after completion of treatment, with combination therapy compared to interferon alone[25-29] (Table 1). Predictors of high rates of SVR include genotypes 2 and 3 (> 80% SVR) and low viral load in children with genotype 1 (< 60000 IU/mL)[25,29]. It is important to note that biochemical and virologic response is accompanied by histologic improvement in patients with SVR in these trials and interferon was well tolerated in children.

As in adults, adverse events related to interferon such as fever, headache, and flu-like symptoms are common during the first weeks of treatment, though appear to be short-lived and less intense in children. Persistent symptoms may include anorexia, weight loss and psychiatric complications such as depression and anxiety. Hematologic abnormalities are also frequent with this combination, including ribavirin-induced anemia, thrombocytopenia, and neutropenia which may require dose adjustment. Growth colony stimulating factor, blood transfusions, or erythropoietin for for treating neutropenia and anemia are rarely recommended in children. Decreases in body weight, growth, BMI related to interferon have been shown to be reversible with cessation of therapy[30].

Two protease inhibitors, boceprevir and telaprevir, were licensed separately in the United States in 2011 for use in combination with pegylated interferon and ribavirin in adults with chronic HCV genotype 1. This triple therapy is associated with a significantly higher rate of sustained virologic response (> 90%) compared with dual therapy alone. Currently, phase 1 and 2 trials are ongoing in children. However, the FDA has halted pediatric studies using Boceprevir and the sponsor for telaprevir has discontinued pediatric enrollment due to the availability of interferon-free options which should be available to children through clinical trials soon.

Led by the recent approval of sofosbuvir, several compounds, including daclatasvir, asunaprevir, and ledipasvir are awaiting expedited FDA approval after initial studies in the adult population with tremendous efficacy and tolerability - including HCV non-responders and relapsers to previous therapy[31]. Advantages of these combinations include a high resistance profile, decreased toxicity, and increased sustained viral response in the absence of interferon. Phase 2 trials with sofosbuvir and ribavirin

Table 2 Orthotopic liver transplants performed from January 1, 2008 through April 30, 2013 for pediatric patients (age 0-17) with a diagnosis of hepatitis C

	2008	2009	2010	2011	2012	2013	Total
Initial OLT	8	3	6	4	7	4	32
Retransplant	0	1	0	0	1	0	2
Total	8	4	6	4	8	4	34

Based on Organ Procurement and Transplantation Network data. OLT: Orthotopic liver transplant.

in children with genotypes 2 and 3 began in 2014. HCV-infected children may soon realize the benefits from the tremendous research in anti-HCV therapy in the last 5 years[32-34].

LIVER TRANSPLANT

Hepatitis C is the most common indication for liver transplantation in US adults but is a rare indication in the pediatric population. Little is known about the natural course of HCV following orthotopic liver transplantation (OLT) in children. The largest study to date evaluating post-transplant outcomes in pediatric patients transplanted for hepatitis C was published by our group in 2006[35]. Sixty-seven children were transplanted for hepatitis C between January 1988 and June 2005 in the United States with a total of 83 grafts. Patient and allograft survivals after the initial transplant were 71.6% and 55%, respectively, at 5 years. Nearly 30% of the patients were listed for retransplantation (the overwhelming majority for HCV recurrence) and 19.3% were ultimately retransplanted. The median time between OLTs for those re-transplanted because of HCV was 290 d. Patient and allograft survival rates decreased to 55.0% and 33.8%, respectively, following retransplantation. At the time of publication, these outcomes were similar to that of adult patients. These data revealed that children can benefit from transplantation but also highlighted our limitations in HCV viral suppression during the post-transplant period and prevention of HCV reinfection.

Children who underwent liver transplant prior to the availability of HCV antibody screening of blood products and donors were at high risk for HCV infection and up to 10.2% developed *de novo* hepatitis C[36]. McDiarmid *et al*[36] evaluated 13 pediatric patients transplanted between 1984 and 1996 with *de novo* hepatitis C. Of these, twelve patients were treated with interferon-2 alpha monotherapy (standard of care at the time the paper was written) and 4 developed rapidly progressive liver failure while on interferon treatment requiring urgent retransplantation. Three of the patients ultimately developed aggressive recurrent HCV after the second OLT and subsequently died from HCV-induced liver failure. In a series published in 2011, Venturi *et al*[37] reported improved outcomes in a group of 10 pediatric patients with *de novo* hepatitis C following pediatric liver transplantation

Table 3 1, 3, and 5-year graft and patient survival rates for deceased donor liver transplants performed for patients with hepatitis C between January 1, 2002 through April 30, 2007

		1-yr (95%CI)	3-yr (95%CI)	5-yr (95%CI)
Pediatric				
	Graft	73.73% (56.73-90.73)	64.52% (46.72-82.31)	52.23% (34.45-70.01)
	Patient	87.69% (72.66-100.0)	83.70% (66.93-100.0)	79.05% (60.34-97.76)
Adult				
	Graft	83.29% (82.58-83.99)	71.58% (70.72-72.43)	64.36% (63.45-65.28)
	Patient	87.29% (86.65-87.93)	76.58% (75.76-77.40)	69.75% (68.85-70.66)

Based on Organ Procurement and Transplantation Network data.

(transplanted between 1985 and 2010). Five patients did not receive antiviral therapy post-OLT - two of which achieved spontaneous viral clearance. Of the 5 patients treated, all received the pegylated form of interferon with ribavirin. Eventually, four achieved SVR and the fifth patient was completing therapy at the time of manuscript publication. All patients were alive with a mean follow-up of 7.3 ± 5.5 years after the diagnosis of HCV. Overall the patients demonstrated a favorable long-term outcome and responded well to treatment.

Since the initiation of routine screening of the blood donor supply in the early 1990's, the number of pediatric patients with hepatitis C requiring liver transplantation has decreased. Per review of UNOS/OPTN Registry data, as of June 2013, there were only 2 pediatric patients awaiting a liver transplant for liver disease secondary to hepatitis C. Between January 2008 and April 2013, only 34 pediatric patients were transplanted with hepatitis C compared to 13754 adults (Table 2). The majority of the pediatric patients were born just prior to the beginning of universal screening of blood products or soon afterwards. Although 1, 3, and 5-year patient survival rates in the pediatric population are better than adult survival rates, the graft survival rates are noticeably reduced (Table 3). This could be due to HCV recurrence prior to the approved use of pegylated interferon or skewed by the small sample size. Overall improved pre and post-transplant care for children and approval of pegylated interferon likely played a role in improved pediatric survival rates.

Liver transplant for children with primary hepatitis C disease is rare and our understanding of the disease in this population is limited. The incidence of hepatitis C in children has decreased since the implementation of routine and effective screening and the number of children requiring liver transplantation for hepatitis C has significantly decreased. In the current era, the treatment goal for pediatric patients with hepatitis C is to prevent progression to end-stage liver disease. Although the current standard of care has remained unchanged for several

years, trials with new regimens are currently ongoing. These combinations are known to have a high resistance profile, decreased toxicity and high rates of cure. Liver transplantation is still the best option for children with end-stage liver disease from hepatitis C and recent data report excellent patient survival rates post-transplant. Although graft survival rates are not as high as in adults, new antiviral therapies to safely and effectively eradicate recurrent HCV following transplant should help increase graft survival rates like never seen before.

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P- Reviewer: Komatsu H **S- Editor**: Gou SX **L- Editor**: A
E- Editor: Ma S

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DOI: 10.3748/wjg.v20.i32.11287

World J Gastroenterol 2014 August 28; 20(32): 11287-11296
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Effect of *GP73* silencing on proliferation and apoptosis in hepatocellular cancer

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Author contributions: Zhang YL, Zhang YC, Han W and Li YM designed the research; Zhang YL, Wang GN, Wei FX, Wang JF and Yuan S performed the research; Jiang JJ and Zhang YW contributed new reagents or analytic tools; Zhang YL and Zhang YC analyzed the data; Zhang YL and Zhang YC wrote the paper.

Supported by The Grant from Gansu Provincial-level Key Laboratory of Digestive System Tumors Open projects Research Fund, No. lzujbky-2011-t03

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Received: December 25, 2013 Revised: February 28, 2014
Accepted: April 21, 2014
Published online: August 28, 2014

Abstract

AIM: To investigate the roles of Golgi protein (GP) 73 in the regulation of cell proliferation and apoptosis.

METHODS: Stealth RNAi targeting *GP73* gene sequence was used to silence its expression in Hep G2 cells and Bel7402 cells. Stealth RNAi effects were assessed by reverse transcriptase polymerase chain reaction and ELISA. Cell proliferation assay and cell cycle analysis were assessed by MTT assay and flow cytometry. Apoptosis was assessed by flow cytometry and transmission electron microscopy. Apoptosis-related proteins were assessed by western immunoblot analysis.

RESULTS: Stealth RNAi targeting *GP73* gene sequence markedly reduced the expression of *GP73* gene. The reduction of GP73 in Hep G2 cells and Bel7402 cells inhibited cell proliferation and induced apoptosis, however, terminal apoptosis occurred in Hep G2 cells, but early apoptosis occurred in Bel7402 cells. Reduced expression of *GP73* gene might lead to a reduction in Bcl-2/Bax ratio, an increase in cytochrome c, but a reduction in capase-3.

CONCLUSION: GP73 might play an important role in proliferation and apoptosis in hepatocellular carcinoma cells.

Key words: Hepatocellular carcinoma; Golgi protein-73; Cell proliferation; Apoptosis

Core tip: Stealth RNAi targeting Golgi protein (GP)73 gene sequence markedly reduced the expression of *GP73* gene. Reduction of GP73 in Hep G2 cells and Bel7402 cells inhibited cell proliferation and induced apoptosis, however, terminal apoptosis occurred in Hep G2 cells, but early apoptosis occurred in Bel7402 cells. Reduced expression of *GP73* gene might lead to a reduction in Bcl-2/ Bax ratio, an increase in cytochrome c, but a reduction in capase-3. GP73 might play an important role in proliferation and apoptosis in Hep G2 and Bel7402 cells.

Zhang YL, Zhang YC, Han W, Li YM, Wang GN, Yuan S, Wei FX, Wang JF, Jiang JJ, Zhang YW. Effect of *GP73* silencing on proliferation and apoptosis in hepatocellular cancer. *World J Gastroenterol* 2014; 20(32): 11287-11296 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11287.htm DOI:

http://dx.doi.org/10.3748/wjg.v20.i32.11287

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide, and > 80% of HCC occurs in developing countries[1]. HCC remains a significant concern of cancer research because of its poor survival rate and high rates of recurrence[2,3]. Prognosis of surgical or loco-regional therapies for patients with intermediate- and advanced-stage disease remains poor[4]. Several effective gene-targeting agents are currently being tested in preclinical studies[5-9]. Unfortunately, no chemotherapy is effective for HCC patients.

Kladney *et al*[10] first isolated Golgi protein (GP)73 in a genetic screen. Expression of GP73 is increased markedly in HCC cells and its serum levels appear to be predictive of HCC[11-15], and several studies have reported the use of GP73 as a serum marker for HCC[16-22]. GP73 may be elevated even when small undetectable tumors are present[23]. The physiological and pathological roles of GP73 have attracted considerable attention in recent years[24-30]. However the function of GP73 in hepatic carcinoma cells remains obscure. The expression of GP73 was silenced in the HCC cell line Bel7402 and Hep G2 by stealth RNAi, which serves as a powerful technology to block specifically the expression of target genes in the present study[31-35]. The effects of GP73 on cell proliferation and apoptosis were also evaluated in this study.

MATERIALS AND METHODS

Stealth RNAi

According to the siRNA design guidelines[27,28], one RNAi target sequence was selected corresponding to the nucleotides of RNAI-Stealth RNAi of the human GP73 mRNA (GenBank Accession No. NM177937.2). The sequence of the synthesized oligonucleotide was: HSS181966: sense 5'-GGAAACGGGCGUCGCAGCAUGAAGU-3', anti-sense 5'-ACUUCAUGC UGCUACGCCCGUUUCC-3'.

Transfection

Lipofectamine RNAi Max transfection agent (Invitrogen, Carlsbad, CA, United States) was used to transfect synthesized Stealth RNAi against GP73 into Hep G2 and Bel7402 cells. BLOCK-iT Alexa Fluor Red Fluorescent (Invitrogen) was used to confirm the transfection efficiency of each duplex siRNA. Stealth RNAi, fluorescent logo or negative control duplexes were delivered into Hep G2 and Bel7402 cells through reverse transfection.

Reverse transcriptase polymerase chain reaction

To analyze quantitatively the effects of Stealth RNAi on GP73 mRNA, cells were transfected with Stealth RNAi or negative control in culture flasks. After 24 and 48 h, cells were harvested by trypsinization and rinsed twice with cold PBS. TRIzol reagent (Invitrogen) was used to extract total RNA. Two-step real-time reverse transcriptase polymerase chain reaction (RT-PCR) kits (TakaRa, Japan) was used to perform first-strand cDNA synthesis and amplification. The 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, United States) was used to perform quantitative PCR amplifications. A 20-μL reaction volume containing 10 μL 2× SYBR Premix Ex TaqTM (TakaRa) was used to carry out this reaction. β-Actin was used as an internal standard. The primer sequences were: GP73 sense 5'-GTGCTGGTGCCAGCCTGTTA-3' and anti-sense 5'-AGTGCTCTAGGCCA TTGATTGATTG-3', β-actin sense 5'-GCAAGCAGGAGTATGACGAGT-3' and anti-sense 5'-GCAAGCAGGAGTATGACGAGT-3'. Thermal cycle conditions: 95 ℃ for 30 s, followed by 40 cycles of 94 ℃ for 5 s, and 61 ℃ for 30 s. The ΔCt of each group was calculated by the formula: $\Delta Ct = Ct_{GP73} - Ct_{\beta\text{-actin}}$. ΔΔCt was calculated by $\Delta Ct_{treated} - \Delta Ct_{control}$. The fold change for GP73 expression levels of the treated groups were calculated using 2-ΔΔCt. The primers described above and the comparative threshold (Ct) method were used to calculate the relative amount of mRNA in the treated samples compared to the control samples. The real-time PCR assays were performed in triplicate.

GP73 proteins detection

To analyze quantitatively the effects of Stealth RNAi on GP73 protein levels in supernatant of Hep G2 and Bel7402 cells, cells were transfected with Stealth RNAi or negative control in culture flasks. After 24 and 48 h, the supernatant was collected, and GP73 protein levels in supernatant of Hep G2 and Bel7402 cells were detected using a commercially available human GP73 ELISA kit. The ELISAs were performed in triplicate.

Cell proliferation assay and cell cycle analysis

Cell proliferation was measured by MTT assay, according to the manufacturer's instructions. Propidium iodide (PI) staining of the nuclei was used to monitor cell cycle assay. Seventy-five percent cold alcohol was used overnight to fix the cells, and then the cells were resuspended in 300 μL PBS and stained with 500 μL PI (250 μg/mL) for 30 min in the dark. Flow cytometry was used to analyze cells. Cell cycle assays were performed in triplicate.

Apoptosis assessment with flow cytometry and transmission electron microscopy

To analyze quantitatively the effects of Stealth RNAi on apoptosis, cells were transfected with Stealth RNAi or negative control in culture flasks. After 48 h, cells were harvested by trypsinization and rinsed twice with cold PBS. Cells were resuspended in 200 μL binding buffer and then treated with 10 μL Annexin V-FITC and 5 μL PI (Sigma, St Louis, MO, United States) for 15 min. Flow cytometric analysis of cells was performed with an EpicsXL Coulter flow cytometer (Beckman-Coulter, United States). All assays were repeated three times.

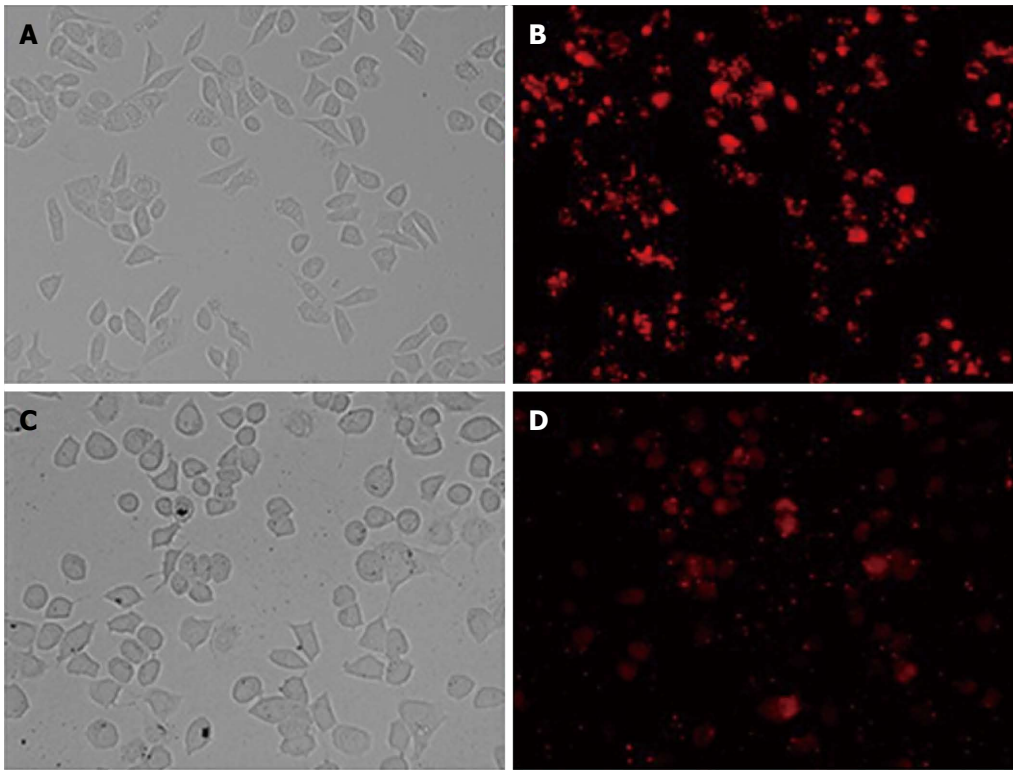

Figure 1 BLOCK-iT Alexa Fluor red fluorescent control. The BLOCK-iT Alexa Fluor red fluorescent control (30 nmol/L) was transfected into Hep G2 and Bel7402 cells using Lipofectamine RNAi MAX Transfection Reagent. Twenty-four hours after transfection, growth medium was removed and replaced with PBS. Hep G2 and Bel7402 cell localization of the Alexa Fluor Red Fluorescent control oligo is seen with fluorescent microscopy (B and D, × 200). Over 80% of the cells took up the control oligo and retained a normal morphology, as seen in bright field (A and C, × 200).

Transmission electron microscopy (TEM) was used to detect apoptosis. Cells were harvested by trypsinization and rinsed twice with cold PBS. Glutaraldehyde (0.3%) was used to prefix the cells overnight, and 10 mL/L osmic acid was used to post-fix the cells. The cells were observed under a JEM-1230 transmission electron microscope (Jeol, Japan).

Protein extraction and western immunoblot analysis

To analyze quantitatively the mechanism of Stealth RNAi on apoptosis, Hep G2 cells were transfected with Stealth RNAi or negative control in culture flasks. After 48 h, cells were harvested by trypsinization and rinsed twice with cold PBS. Hep G2 cells (5×10^5) were lysed by lysis buffer (phenylmethylsulfonyl fluoride), then drawing the protein standard curve to calculate the density of total protein. Ten percent SDS-PAGE was used to separate proteins, and proteins were transferred to nitrocellulose membranes. Anti-GP73, Bax, Bcl-2, cytochrome *c* and procaspase-3, β-actin primary antibody (1:1000; Abcam, Cambridge, MA, United States) were used to incubate membranes, and then, anti-rabbit secondary antibody conjugated with horseradish peroxidase was used to incubate membranes (1:5000) again. Western Blotting Substrate (Bio-Rad) was used to visualize immunoreactive proteins. All assays were repeated three times.

Statistical analysis

Mean ± SD was used to express the data. The data among the three groups were compared by one-way analysis of variance followed by Bonferroni correction. Some data were also analyzed by Student's *t* test. SPSS for Windows version 19.0 was used for statistical analysis. $P < 0.01$ was considered significant.

RESULTS

Transfection efficiency

Twenty-four hours after transfection, Bel7402 and Hep G2 cells were observed by fluorescent microscopy. The efficiency of transfection was > 80% when using 30 nmol/L final concentrations of oligo duplex and 1×10^5/mL cells (Figure 1). Therefore, 30 nmol/L siRNA and 1×10^5/mL Bel7402 and Hep G2 cells were used to perform subsequent experiments.

Expression of GP73 after transfection

After Stealth siRNA targeting GP73 was transfected into Hep G2 and Bel7402 cells, mRNA levels were measured by RT-PCR. There was an obvious reduction of GP73 mRNA levels in the Stealth RNAi group. Quantification analysis revealed that GP73 mRNA was reduced by 68.7% and 90.3% of the blank control for Hep G2 cells, and 56.7% and 88.7% of the blank control for Bel7402 cells at 24 and 48 h after transfection, respectively. A significant difference was found between the blank control and Stealth RNAi groups (Figure 2A and B, $P < 0.01$). ELISA of supernatant demonstrated that GP73 protein

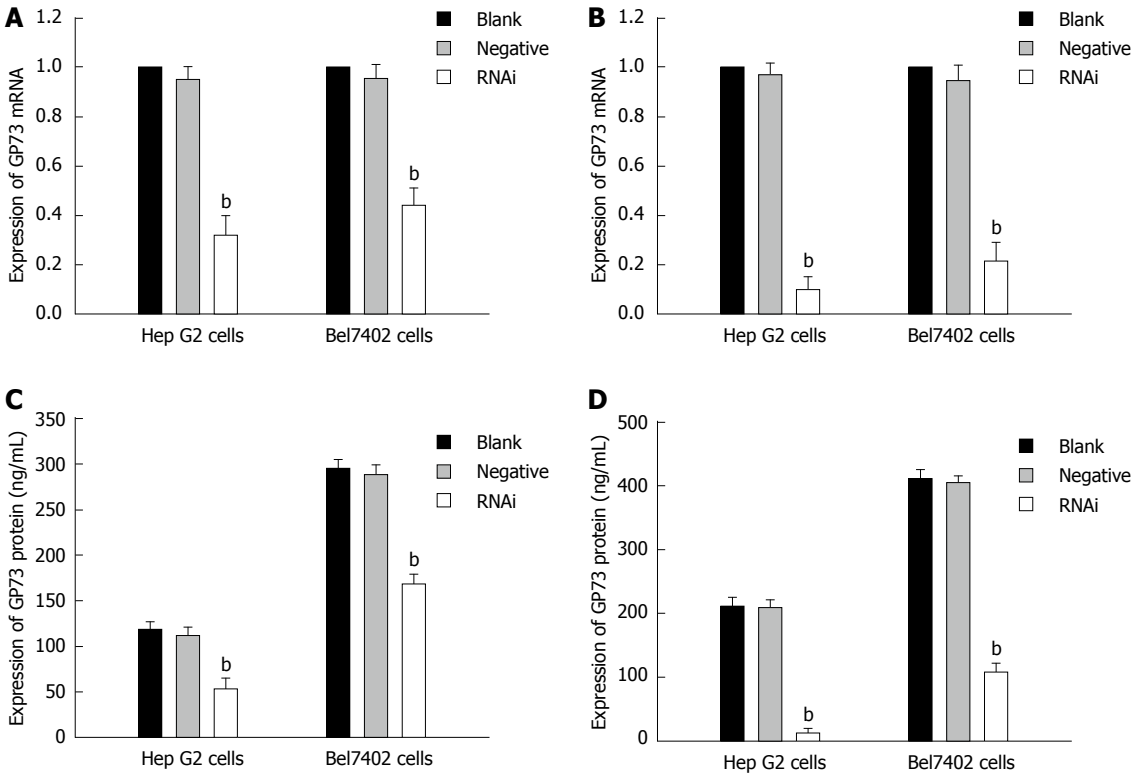

Figure 2 Expression of GP73 mRNA and protein for Hep G2 and Bel7402 cells after transfection with Stealth RNAi against GP73, negative control RNAi or blank control. A: Relative expression of GP73 mRNA level at 24 h after transfection analyzed by quantitative real-time reverse transcription polymerase chain reaction (RT-PCR); B: Relative expression of GP73 mRNA level at 48 h after transfection analyzed by quantitative RT-PCR; C: Expression of GP73 proteins in supernatant of Hep G2 and Bel7402 cells were detected by enzyme-linked immunosorbent assay (ELISA) at 24 and 48 h; D: Expression of GP73 proteins in supernatant of Hep G2 and Bel7402 cells was detected by ELISA at 48 h. The corresponding values of GP73 protein in the three groups at 24 and 48 h after transfection. $^{b}P < 0.01$ *vs* blank group.

levels were also significantly reduced compared with the blank control. GP73 protein levels for blank control, negative control and RNAi groups at 24 and 48 h after transfection are shown in Figure 2C and D ($P < 0.01$). There was a significant difference between the blank control and RNAi groups (Figure 2C and D, $P < 0.01$). Expression of GP73 in Hep G2 cells was also significantly lower than in Bel7402 cells after transfection.

Silencing of GP73 gene decreased viability and proliferation in Bel7402 and Hep G2 cells

As illustrated in Figure 3A, the viability of Hep G2 and Bel7402 cells was reduced significantly after transfection. The inhibition rate in the RNAi group was 27.09% and 50.53% at 24 and 48 h after transfection for Hep G2 cells, respectively, and 21.3% and 46.4% at 24 and 48 h after transfection for Bel7402 cells (Figure 3A, $P < 0.01$). As illustrated in Figure 3B and C, an apoptotic peak was seen in the RNAi group of Hep G2 cells, and cell cycle analysis showed that proliferation of Hep G2 cells yielded 78.22% ± 0.35% cells in the G_0/G_1 phase, 20.72% ± 1.19% cells in S phase, and 1.07% ± 1.09% cells in G_2/M phase 48 h after transfection. The percentage of cells in G_0/G_1 phase increased significantly, however, the percentage of cells in G_2/M phase decreased significantly ($P < 0.01$). Proliferation control Bel7402 cells yielded 88.81% ± 1.13% cells in G_0/G_1 phase, 12.1% ± 1.12% cells in S phase, and 0.98% ± 1.08% cells in G_2/M phase 48 h after transfection in the RNAi group. The percentage of cells in G_0/G_1 phase increased significantly, however, the percentage of cells in G_2/M phase decreased significantly in both cells.

Silencing of GP73 gene induces apoptosis

After transfection for 48 h, the apoptotic cells were markedly increased in the RNAi group compared to those in the negative and blank control groups in Hep G2 and Bel7402 cells. Their values are shown in Figure 4 ($P < 0.01$). The results showed that terminal- and early-stage apoptotic cells were markedly increased in the RNAi group compared to the blank control group in both cells, and terminal-stage apoptosis mainly occurred in Hep G2 cells after transfection, however, early-stage apoptosis mainly occurred in Bel7402 cells.

Changes in ultrastructural morphology of cells

Normal control Hep G2 cells were observed by TEM (Figure 5A). Some typical manifestations of apoptosis such as vacuoles, nuclear fragmentation and apoptotic bodies were observed in the RNAi group of Hep G2 cells (Figure 5B). Normal control Bel7402 cells were observed (Figure 5C). Dense chromatin and some vacuoles could

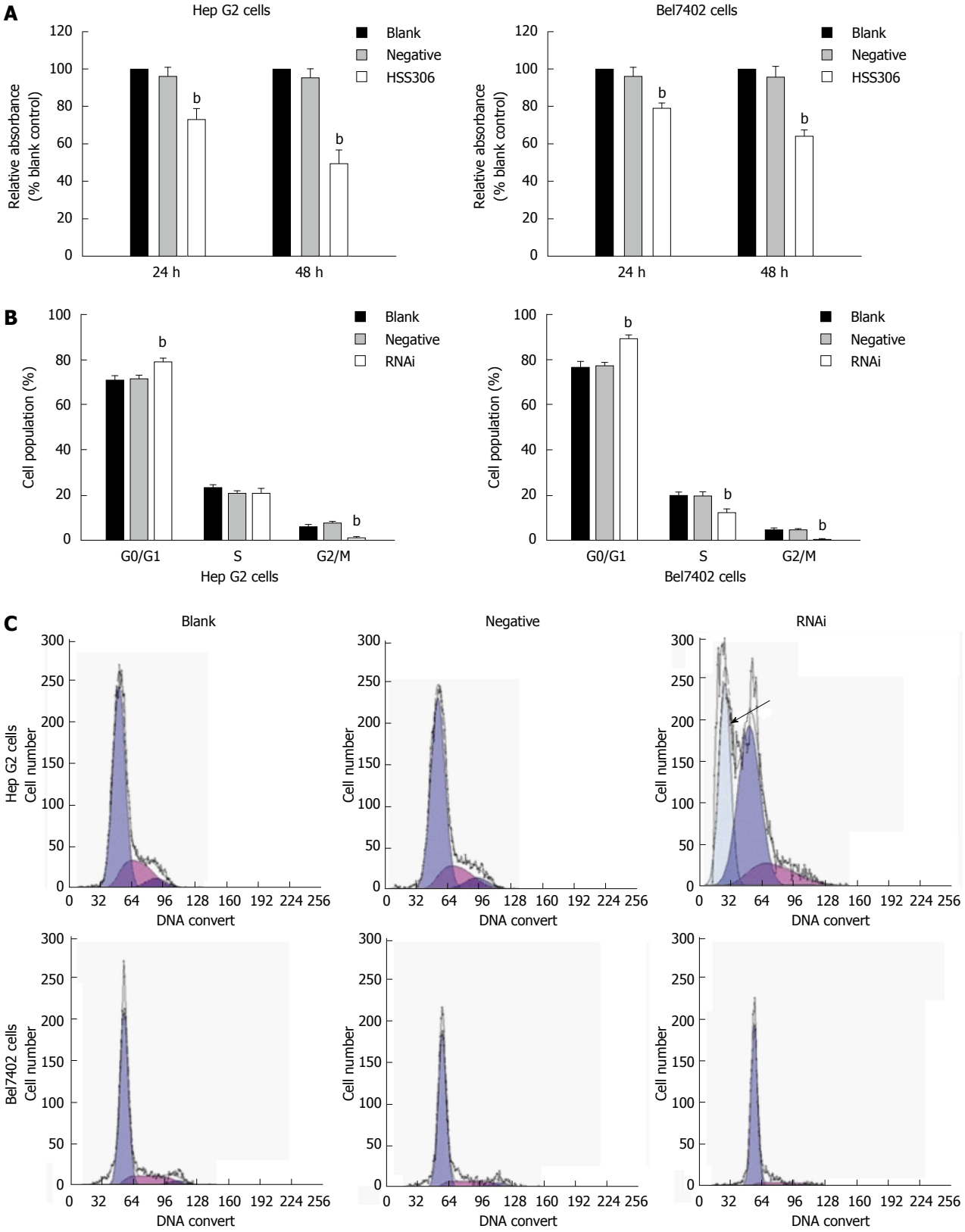

Figure 3 Silencing of *GP73* gene decreased cell proliferation. A: MTT analysis at 24 and 48 h (% blank control) for Hep G2 and Bel7402 cells; B: Cell cycle assay was analyzed by flow cytometry; C: Flow cytometric analysis of the cell cycle distribution of Hep G2 and Bel7402 cells at 24 h after transfection. Cells were washed, fixed, and stained with propidium iodide, and analyzed using Beckman-Coulter Epics flow cytometer. $^{b}P < 0.01$ *vs* blank group.

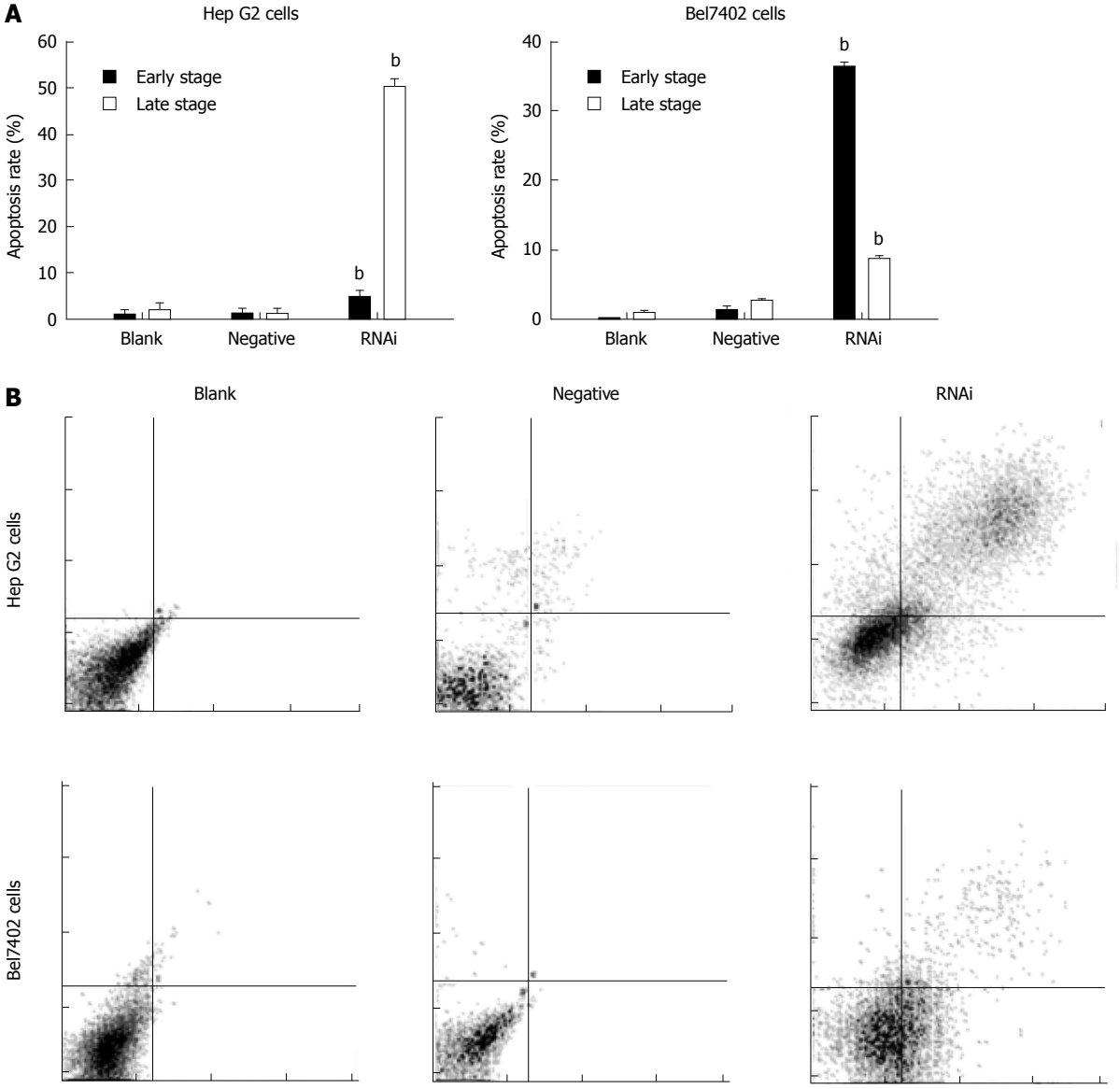

Figure 4 Effect of GP73-targeted stealth RNAi transfection on apoptosis of Hep G2 and Bel7402 cells. A: Apoptosis assay was analyzed by flow cytometry; B: Apoptotic effect of Stealth RNAi after 48 h, and later stained with annexin V-FITC and PI. The stained cells were analyzed for apoptosis using a Beckman-Coulter Epics flow cytometer. $^{b}P < 0.01$ *vs* blank group.

be seen in the RNAi group of Bel7402 cells (Figure 5D).

Effects of silencing GP73 gene on apoptosis-related molecules in Hep G2 cells

Western blotting was performed to analyze the expression of GP73, Bax/Bcl-2, cytochrome c and procaspase-3 in Hep G2 cells. The protein levels of GP73 decreased significantly compared with those in negative and blank control groups at 48 h after transfection in Hep G2 cells. Compared with negative and blank control groups, the protein levels of Bcl-2 were reduced significantly, however, the protein levels of Bax were markedly elevated. Compared to the negative and blank control groups at 48 h after transfection, the protein level of cytochrome c in the cytoplasm was markedly elevated in the RNAi group, and the protein level of procaspase-3 was reduced markedly (Figure 6).

DISCUSSION

There is a growing body of evidence that GP73 serum levels correlate with the presence of HCC. However, although GP73 levels in the circulation do not appear to be elevated in healthy subjects, these and other reports suggest elevation of the marker in people with inflammatory liver diseases, in the absence of cancer. Thus, its usefulness in distinguishing cancer from cirrhosis or other liver conditions associated with inflammation is still being evaluated. Work by others has found that GP73 levels are affected by tumor necrosis factor-α and interferon-γ in tissue culture systems, and there is an association between levels of GP73 and osmoles in serum from people with liver cirrhosis, but there is no significant correlation in HCC. We do not know the molecular basis for the high expression of GP73 in these cells, and the effect

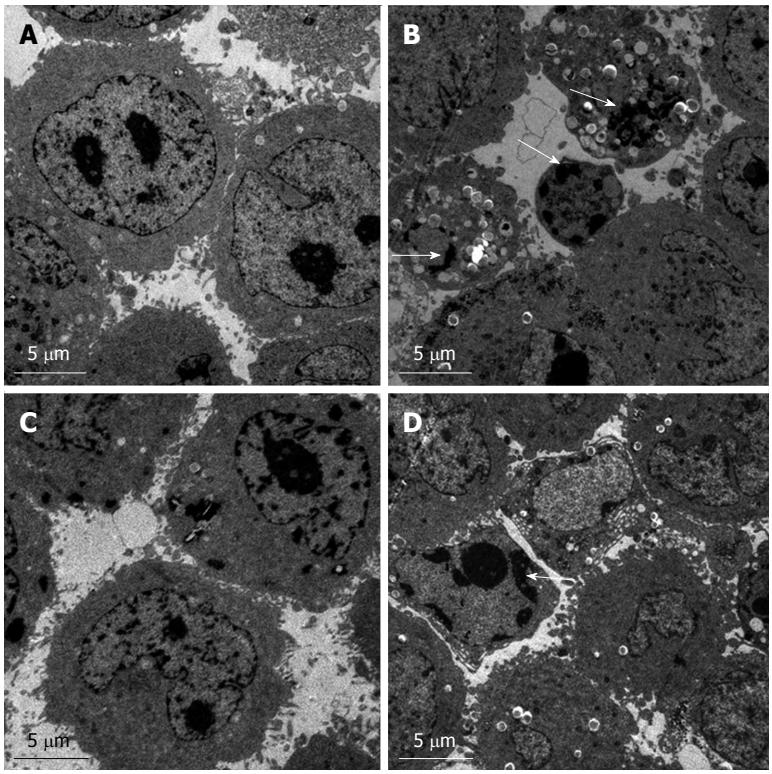

Figure 5 Transmission electron microscopy. The ultrastructural morphology of control (A) and RNAi (B) of Hep G2 cells. The ultrastructural morphology of control (C) and RNAi (D) Bel7402 cells. Nuclear fragmentation and apoptotic bodies are indicated by arrows.

of GP73 in hepatic carcinoma cells is also unclear. We investigated the effect of GP73 in hepatic carcinoma by silencing expression of GP73 through siRNA. In this study, after Stealth RNAi against GP73 was transfected into Hep G2 and Bel7402 cells, the expression of GP73 and mRNA of GP73 decreased significantly in both cells. The decreasing expression of GP73 inhibited cell proliferation and led to apoptosis. Besides, decreasing expression of GP73 also leads to reduction of Bcl-2/Bax ratio, an increase in protein level of cytochrome c in the cytoplasm, and decreasing procaspase-3.

The effects of Stealth RNAi on both GP73 mRNA and protein levels were measured. The results demonstrated that the Stealth RNAi against GP73 (RNAi) can reduce the expression of GP73 effectively at both mRNA and protein levels in Hep G2 and Bel7402 cells after transfection. Western blotting showed that GP73 protein decreased significantly in the RNAi group compared with the other groups. In addition, cell cycle assay confirmed that the proliferation of Bel7402 and Hep G2 cells was reduced significantly after expression of GP73 gene was silenced by RNAi. In Bel7402 and Hep G2 cells, the percentage of cells in G_0/G_1 phase increased significantly, however, the percentage of cells in G_2/M phase decreased significantly. The percentage of cells in S phase decreased in Bel7402 cells after transfection, although the percentage of Hep G2 cells in S phase did not change significantly. However, an apoptotic peak was seen in Hep G2 cells, which indicates that terminal apoptosis had occurred. The proliferation of hepatic cancer cells is related to the expression of GP73. Decreasing expression of GP73 inhibited hepatic cancer cells in G_0/G_1 phase, and sometimes it also affected hepatic cancer cells in S phase.

Furthermore, fluorescence activated cell sorting methods suggested that Stealth RNAi against GP73 (RNAi) caused accumulation of early-stage apoptotic Bel7402 cells and accumulation of terminal-stage apoptotic Hep G2 cells at 48 h after transfection (GP73 was silenced). The apoptotic peak was also seen in proliferation of Hep G2 cells. The different stage of apoptosis may be related to the different expression of GP73 in Bel7402 and Hep G2 cells. TEM observation agreed with the former results. Dense chromatin appeared near the nuclear membrane, many foaming phenomenonhappened in Bel7402 cells, which also indicated that Stealth RNAi against GP73 (RNAi) mainly caused accumulation of early-stage apoptotic Bel7402 cells, however, many vacuoles, nuclear fragmentation and apoptotic bodies appeared in Hep G2 cells, which can also explain why there was an apoptotic peak in the RNAi group. These results also confirmed that Stealth RNAi against GP73 (RNAi) mainly caused accumulation of terminal-stage apoptotic Hep G2 cells. And the difference of its expression in different cells might play different function. Several studies have found that the ratio of Bax/Bcl-2 is important in apoptosis of cancer cells. Bcl-2 is considered as an upstream effector molecule in the apoptotic pathway, but Bax is considered as a downstream effector molecule[36,37]. The protein levels of Bax and Bcl-2 were measured, and similar results

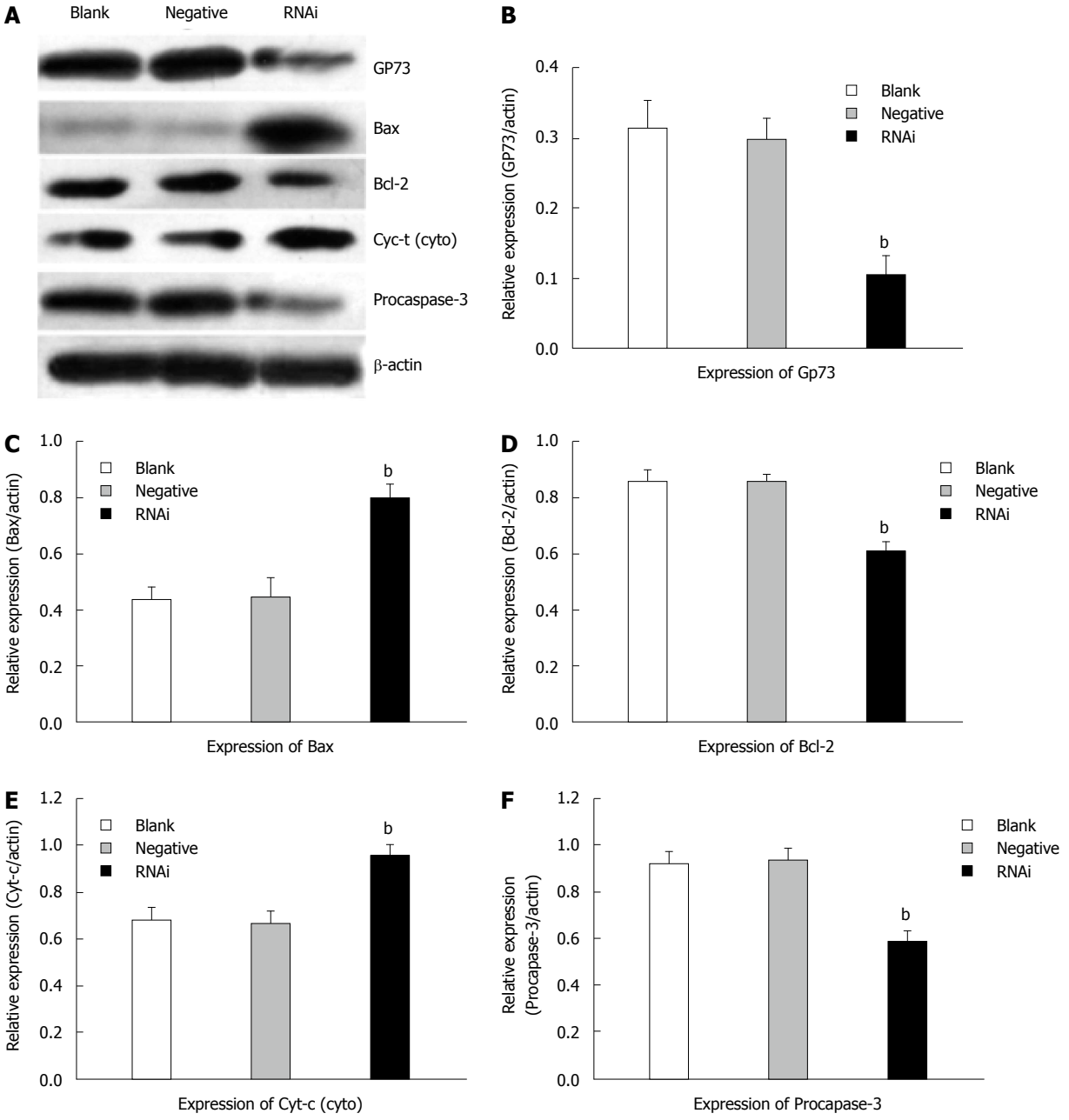

Figure 6 Effects of silencing *GP73* gene on apoptosis-related molecules in Hep G2 cells. A: Western blotting detected expression of GP73, Bcl-2, Bax, cytochrome c and procaspase-3 in Hep G2 cells at 48 h after transfection with Stealth RNAi (HSS181966; Stealth RNAi against GP73, negative control RNAi) or blank control; B-F: Representative immunoblots are shown from three independent experiments. Software Glyko Bandscan 5.0 was used to quantify the bands, and the expression of β-actin was used as a loading control, the protein levels were normalized against β-actin, data were expressed as mean ± SD ($^bP < 0.01$ *vs* blank group).

were also found in this study. We demonstrated that the reduction of GP73 led to a decrease in expression of Bcl-2 but an increase in Bax, and the Bcl-2/Bax ratio in Hep G2 cells was reduced, this result was also similar to the other one on the cell apoptotic mechanism[38]. Besides, in some apoptotic mechanism studies, it was found that Bcl-2 inhibits cytochrome c release from mitochondria, whereas Bax triggers it[39], and release of cytochrome c activated caspases to induce apoptosis. The expression of cytochrome c and procaspase-3 was also detected by Western blotting to determine their distribution in apoptosis. The level of cytochrome c in the cytoplasm was markedly elevated in the RNAi group; however, the level of procaspase-3 was reduced significantly. These results proved that GP73 may play a major role in anti-apoptosis in HCC cells, and there is a correlation between it and other apoptosis-related proteins such as Bax, Bcl-2, cytochrome c and procaspase-3.

In conclusion, Stealth RNAi targeting GP73 gene sequence reduced the expression of GP73 markedly. The reduction of GP73 in Hep G2 and Bel7402 cells inhibited cell proliferation and induced apoptosis, however, terminal apoptosis occurred in Hep G2 cells, but early apoptosis occurred in Bel7402 cells. Reduced expression

of *GP73* gene might lead to a reduction in Bcl-2/Bax ratio, an increase in cytochrome c, but a reduction in capase-3, and the reduction of GP73 induces apoptosis in Hep G2 cells may through this apoptosis signaling pathway. However, further studies are needed to confirm this conclusion. Consequently, the results imply that GP73 plays an important role in HCC cells, but unfortunately, expression of GP73 was not reduced to a low level in this study, therefore, more effective molecular tools are needed to explore the exact function of GP73 in more HCC cells.

COMMENTS

Background

The physiological and pathological roles of Golgi protein (GP)73 have attracted considerable attention in recent years. However, the function of GP73 in hepatic carcinoma cells remains obscure.

Research frontiers

This study was performed to explore the function of GP73 in hepatic carcinoma cells; Stealth RNAi targeting *GP73* gene sequence was used to silence its expression; and cell proliferation and apoptosis were assessed. This study implied that GP73 plays an important role in hepatocellular carcinoma (HCC) cells.

Innovations and breakthroughs

The results of this study suggested that silencing *GP73* gene in HCC cells inhibits cell proliferation and induces apoptosis.

Applications

The results imply that GP73 plays an important role in HCC cells, but further research is needed to explore the exact function of GP73 in more HCC cells.

Peer review

These results are very interesting and may provide important evidence about the mechanism of apoptosis in HCC.

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P- Reviewer: Chuma M, Smith RC **S- Editor**: Gou SX
L- Editor: Kerr C **E- Editor**: Ma S

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DOI: 10.3748/wjg.v20.i32.11297

World J Gastroenterol 2014 August 28; 20(32): 11297-11304
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Role of moxibustion in inflammatory responses during treatment of rat ulcerative colitis

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Supported by Scientific and Technological Project of Educational Department of Liaoning Province, China, No. L2011166
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Received: March 20, 2014 Revised: May 30, 2014
Accepted: July 16, 2014
Published online: August 28, 2014

Abstract

AIM: To investigate the efficacy of moxibustion in ulcerative colitis (UC) rats from morphological, immunological and molecular biological perspectives.

METHODS: Thirty-two Sprague-Dawley rats were randomly assigned to a blank control group (normal rats, n = 6) and a model replication (MR) group (UC rats, n = 26). A UC model was established by 2,4,6-trinitrobenzenesulfonic acid/dextran sulfate sodium enema. Rats in the MR group were further randomly assigned to a 9-min moxibustion (9M) group (9 moxa-cone, n = 6), 6-min moxibustion (6M) group (6 moxa-cone, n = 6), 3-min moxibustion (3M) group (3 moxa-cone, n = 6), and a waiting list control (WLC) group (no moxibustion treatment, n = 6). Rats in the moxibustion treatment group were treated in 14 sessions over 28 d. Disease activity, local tissue morphology, serum level of interleukin (IL)-8 and IL-10, and expression of Toll-like receptor (TLR)9 as well as nuclear factor (NF)-κB p65 in colonic tissue were determined by disease activity index (DAI), hematoxylin and eosin staining, electron microscopy, enzyme-linked immunosorbent assay and Western blotting, respectively.

RESULTS: DAI was lowest in the 9M group and highest in the WLC group. The differences in DAI between the moxibustion treatment (3M, 6M, 9M) and no treatment groups were significant for all one-to-one comparisons (0.60 ± 0.54 *vs* 1.20 ± 0.44, 0.60 ± 0.54 *vs* 1.80 ± 0.45, 0.60 ± 0.54 *vs* 3.0 ± 0.45, respectively, $P < 0.05$). Light and electron microscopy showed that the neatness of the glandular arrangement in colonic mucosal epithelia gradually increased in the WLC, 3M, 6M to 9M groups. IL-8 level successively decreased while IL-10 level increased from the WLC to 3M, 6M and 9M groups. The differences among these groups were significant for all comparisons (105.46 ± 8.75 *vs* 76.61 ± 3.58, 105.46 ± 8.75 *vs* 69.78 ± 1.87, 105.46 ± 8.75 *vs* 67.41 ± 1.84, respectively, $P < 0.01$ for IL-8; and 30.83 ± 1.29 *vs* 75.64 ± 1.90, 30.83 ± 1.29 *vs* 80.90 ± 3.16, 30.83 ± 1.29 *vs* 83.46 ± 2.37, respectively, $P < 0.01$ for IL-10), except comparison of 6M *vs* 9M. Expression of TLR9 and NF-κB p65 decreased in order: highest in the WLC group and lowest in the 9M group. In addition, the differences among the WLC, 3M, 6M and 9M groups were significant for all comparisons (0.492 ± 0.026 *vs* 0.380 ± 0.022, 0.492 ± 0.026 *vs* 0.355 ± 0.005, 0.492 ± 0.026 *vs* 0.327 ± 0.015, respectively, $P < 0.05$ for TLR9; and 0.436 ± 0.041 *vs* 0.326 ± 0.022, 0.436 ± 0.041 *vs* 0.293 ± 0.006, 0.436 ± 0.041 *vs* 0.265 ± 0.017, respectively, $P < 0.05$ for NF-κB p65).

CONCLUSION: Moxibustion repairs damaged colonic mucosa, suppresses serum IL-8, activates serum IL-10 level, and decreases expression of TLR-9 and NF-κB p65 in UC rats.

Key words: Moxibustion; Ulcerative colitis; Disease activity index; Interleukin-8; Interleukin-10; Toll-like receptor

9; Nuclear factor-κB p65

Core tip: We investigated the effectiveness of moxibustion treatment in ulcerative colitis rats from a modern medicine perspective. In addition, we correlated the effects of moxibustion therapy with immune or inflammatory responses by observing levels of interleukin (IL)-8, IL-10, Toll-like receptor 9, and nuclear factor-κB p65, and the underlying mechanisms were suggested.

Han Y, Ma TM, Lu ML, Ren L, Ma XD, Bai ZH. Role of moxibustion in inflammatory responses during treatment of rat ulcerative colitis. *World J Gastroenterol* 2014; 20(32): 11297-11304 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11297.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11297

INTRODUCTION

Moxibustion (form of acupuncture point stimulation using heat) is a traditional healing technique that is a useful and important therapy in Asian medicine, including in China, South Korea, and Japan[1-4]. In the western world, moxibustion and acupuncture are gaining more attention as alternative and complementary therapeutic interventions, due to relatively low clinical side effects and a higher compliance in patients compared to drug therapy or surgical procedures[5-8]. Recently, acupuncture and moxibustion have been used worldwide as alternative treatments for chronic back pain[9-11], asthma[12,13], stroke rehabilitation[8,14,15], and gastrointestinal disease[7,16].

Ulcerative colitis (UC) is a nonspecific inflammatory bowel disease that afflicts millions of people worldwide. The pathogenesis of UC mainly involves erosions and ulcers with common clinical manifestations of diarrhea, weight loss, abdominal pain, bloody stools, fever, and fatigue[17]. Although the exact cause of UC remains uncertain, most scientists and scholars agree on a combination of genetic and environmental factors. On the basis of various genetic abnormalities, an immune reaction is triggered by environmental factors that transiently break the mucosal barrier with abnormal responses to pathogenic enteric bacteria, and further give rise to onset and reactivation of disease[17-19]. The pathogenesis of UC is associated with immunological abnormalities, and various factors involved in the immune system may directly/indirectly relate to UC. For instance, two inflammatory mediators, interleukin (IL)-8 (neutrophil chemotactic factor) and IL-10 (human cytokine synthesis inhibitory factor) are genetically linked to the inflammation of UC[20,21]. Toll-like receptor (TLR) 9 and nuclear factor (NF)-κB p65 are also intrinsically associated with the inflammatory reaction of UC[22,23].

In addition to the above research on UC from a modern medicine perspective, there is also some evidence that moxibustion and acupuncture are effective in UC. For example, Zhang[24] found that UC symptoms are improved after moxibustion or acupuncture. Ma[25] showed that moxibustion or acupuncture was effective for treating UC by observing the symptoms in 76 patients. Joos *et al*[6] have suggested that acupuncture offers an additional therapeutic benefit in patients with mild to moderately active UC. Most research has focused on whether moxibustion and acupuncture are effective, and only rarely has it investigated the mechanistic connection between moxibustion or acupuncture therapy and drug therapy. Wu *et al*[26] have suggested that moxibustion and acupuncture inhibit expression of inflammatory cytokines by observing IL-1β and IL-6 mRNA expression in the spleen and colonic mucosa of UC rats. Although their work investigated the mechanisms of moxibustion or acupuncture treatment for UC, their results were limited to qualitative observation of electrophoresis patterns. Further research, such as quantitative research, is necessary to shed some light on the role of moxibustion and acupuncture in the treatment of UC, and to elucidate why moxibustion and acupuncture achieve their therapeutic effects.

In this study, we quantitatively investigated the efficacy of moxibustion in the treatment of UC rats by observing changes in physical and biological parameters [disease activity index (DAI), colonic epithelial glandular arrangement, levels of IL-8, IL-10, TLR9, and NF-κB P65]. The purpose of this study was to obtain quantitative information about the efficacy of moxibustion to provide useful information for future clinical practice. In addition, we investigated a comprehensive theoretical system of moxibustion treatment for UC by providing direct scientific evidence of its therapeutic effects from a modern medicine perspective.

MATERIALS AND METHODS

Materials

Thirty-two Sprague-Dawley rats (150 ± 20 g, 16 female and 16 male) were provided by the Laboratory Animal Center of China Medical University. Rat IL-8 and IL-10 ELISA kits were purchased from Shanghai Yueyan (China). Rat anti-mouse TLR9 antibody and rat anti-mouse NF-κB p65 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, United States). Goat anti-goat anti-rabbit IgG/HRP antibody was purchased from Beijing Biosynthesis Biotechnology Co. Ltd. (China). Protein Miniprep kit was purchased from Tiandz (Beijing, China). Protein assay kit was purchased from Beyotine Institute of Biotechnology (Shanghai, China).

Rat model of UC

The rat model of UC was established using an immunological method. Acute colitis was induced by rectal administration of 100 mg/kg 2,4,6-trinitrobenzenesulfonic acid in 50% ethanol. After the fecal occult blood test showed "1+", rats with acute colitis were treated with 5% dextran sulfate sodium solution or normal saline to induce inflammation for the second time. UC was induced after 21 d. Colonic tissue stained by HE was examined by light microscopy (Olympus BX41, Tokyo, Japan). Morphology of colonic mucosa was studied by electron microscopy (Olympus CHA). The colonic tissue and

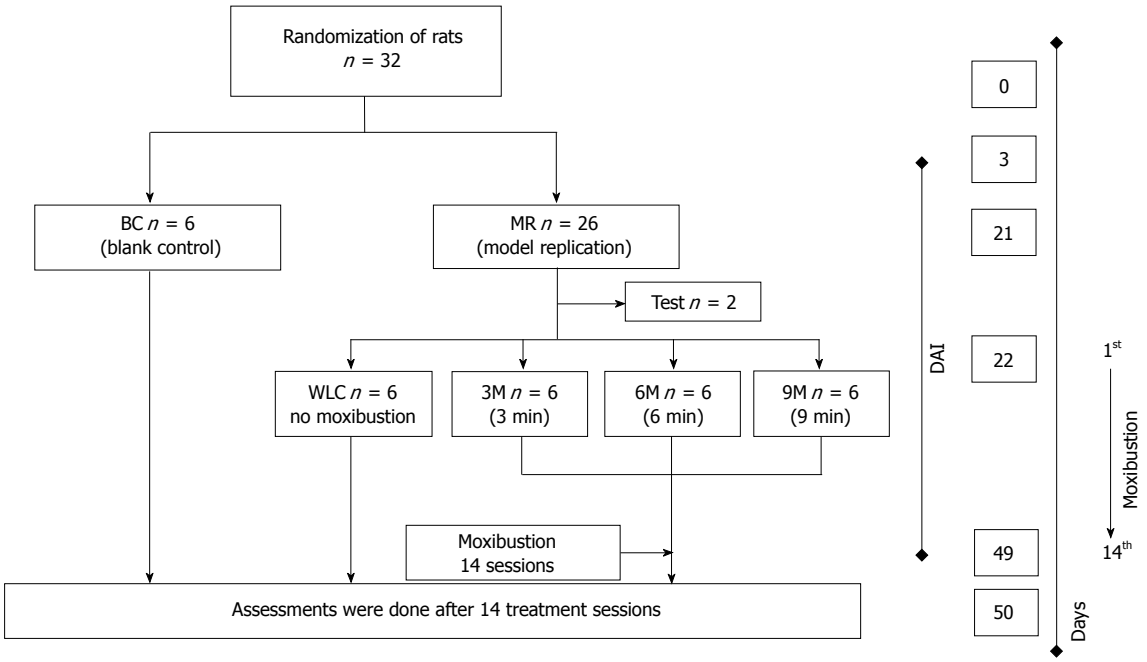


Figure 1 Study design of moxibustion treatment in ulcerative colitis rats. Blank control (BC) indicates control group with normal rats. Model replication (MR) is rat model of ulcerative colitis (UC). Waiting list control (WLC) is the control group in which UC rats did not receive moxibustion treatment. 3-min moxibustion (3M), 6-min moxibustion (6M) and 9-min moxibustion (9M) are 3-moxa, 6-moxa, and 9-moxa groups in which UC rats received different durations of moxibustion (3, 6 and 9 min) in each session (2 d). The whole moxibustion treatment course included 14 sessions from days 22 to 49. DAI: Disease activity index.

Table 1 Ulcerative colitis disease activity index

Rate of body mass loss	Fecal viscosity	Rectal bleeding	Rating
None	Normal	None	0
1%-5%	Low to medium	Concealed hemorrhage	1
5%-10%	Medium	Concealed hemorrhage	2
10%-15%	Slightly low	Revealed hemorrhage	3
> 15%	Low	Revealed hemorrhage	4

mucosal epithelium indicated successful establishment of UC.

Moxibustion treatment

Home-made moxa cones (refined mugwort floss: 6 mm in diameter, 7 mm in base diameter, 8 mm in height, 50 mg in weight) were placed on *Daheng* (SP15, bilateral) and *Tianshu* (ST25, bilateral) and ignited. UC rats received moxibustion treatment for 3 min (3M group), 6 min (6M group), and 9 min (9M group). Three, six and nine moxa cones were used for each treatment session in the 3M, 6M and 9M groups. The whole treatment course comprised 14 sessions.

Study design

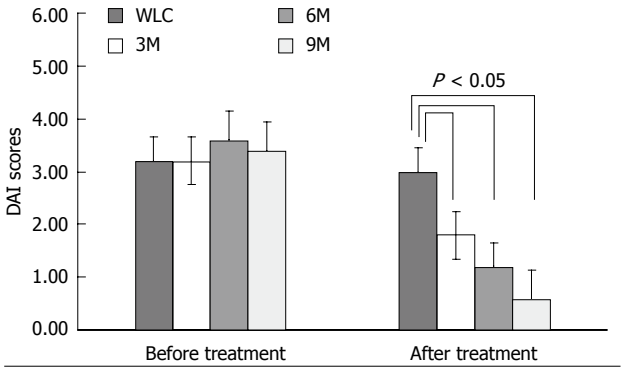
Figure 1 shows the study design for moxibustion treatment of UC rats. Thirty-two normal rats (16 male and 16 female) were randomly assigned to two groups. One was named the blank control (BC) group and consisted of six normal rats without moxibustion treatment. The other was the model replication (MR) group that consisted of 26 rats with UC. Establishment of the UC model lasted 21 d. After that, two rats were randomly selected to determine whether UC had been induced. The observation of disease activity, reflected in DAI, started from day 3 and ended after the moxibustion course. After UC was induced, the MR group was further randomly assigned into three subgroups (3M, 6M and 9M) with six rats each. 3M, 6M and 9M were named according to the timespan of moxibustion treatment in each session, which was 3, 6 and 9 min, respectively. Each session lasted 2 d, in which UC rats only received moxibustion treatment. The whole moxibustion course included 14 sessions (28 d in total). After the entire treatment course, all rats were killed simultaneously. The assessments including IL-8, IL-10, TLR9, and NF-κB p65 measurement were conducted on the next day.

DAI

The disease activity of UC rats was observed from day 3 to the last day of 14 moxibustion sessions (Figure 1) and was evaluated based on the index shown in Table 1. The index assessed four variables, which included rate of body mass loss, fecal viscosity, rectal bleeding, and the corresponding disease activity in terms of each variable. Normal fecal viscosity means solid shaped feces, medium fecal viscosity indicates semi-formed feces, and low fecal viscosity represents watery loose feces that can attach to the anus. The physician's overall assessment of disease activity was determined by the average rating of body mass loss, fecal viscosity and rectal bleeding, which is expressed as the equation: DAI = (body mass loss + fecal viscosity + rectal bleeding)/3.

Detection of IL-8, IL-10, TLR9 and NF-κB p65

The levels of IL-8 and IL-10 in the serum were detected



	Before treatment	After treatment
WLC	3.20	3.00
3M	3.20	1.80
6M	3.60	1.20
9M	3.40	0.60

Figure 2 Comparison of disease activity index in ulcerative colitis rats before and after moxibustion treatment. Ulcerative colitis (UC) rats in the waiting list control (WLC) group did not receive moxibustion treatment. UC rats in the 3-min moxibustion (3M), 6-min moxibustion (6M) and 9-min moxibustion (9M) groups received 3, 6 and 9 min, respectively, in each treatment session. Values are the average of 14 sessions. Before treatment, there was no significant difference in disease activity index (DAI) among the groups. After treatment, significant differences ($P < 0.05$) in disease activity were found. In the 3M, 6M and 9M groups, DAI markedly decreased after moxibustion treatment. Error bar represents SD, and significance was assessed using least significant difference.

by typical enzyme-linked immunosorbent assay, according to the manufacturer's instructions (Shanghai Yueyan, China). Expression of TLR9 and NF-κB p65 in the colonic tissue was detected by western blotting, according to the protocol available in the Protein Miniprep kit and protein assay kit.

Statistical analysis

Data analysis was performed using SPSS version 12 software. Least significant difference was used to evaluate the differential efficacy of moxibustion treatment in UC rats. P values < 0.05 (two-sided) were regarded as statistically significant.

RESULTS

Disease activity observation

DAI has been widely used for the evaluation of disease activity, allowing the integration of various aspects of the disease into a single value[27,28]. To investigate whether moxibustion is effective in improving the disease activity of UC rats, we evaluated the DAI by continuously observing the rate of body mass loss, fecal viscosity, and rectal bleeding for 14 sessions with 2 d/session. Figure 2 shows the DAI of UC rats in the different groups before and after moxibustion treatment, and comparison of disease activity between the moxibustion-treated rats (3M, 6M and 9M groups) and moxibustion-free rats [waiting list control (WLC) group]. The values given were the average of DAI scores obtained in 14 sessions. From the pre-treatment data, DAI was relatively static among the different groups (3.20 for WLC, 3.20 for 3M, 3.60 for 6M, and 3.40 for 9M), which indicated the legitimacy of sampling randomization. In contrast, DAI represented a significant change between each moxibustion group and the moxibustion-free group (3M *vs* WLC, 6M *vs* WLC, and 9M *vs* WLC, all $P < 0.05$). The significant decrease in DAI between the moxibustion-free and moxibustion-treated groups indicated the efficacy of moxibustion in UC rats. In the moxibustion-treated groups, the duration of treatment gradually increased from 3 to 6 and 9 min. We also noticed that DAI decreased from 1.80 to 0.60 as the duration of treatment increased from 3 to 9 min. This implies that 9 min moxibustion probably yields better improvement of UC disease activity, while taking physical tolerance into account.

Observation of colonic tissue and mucosal epithelia

In order to visualize the efficacy of moxibustion treatment in UC rats, we compared the condition of colonic tissue stained by HE and observed by light microscopy (Figure 3A). For normal rats in the BC group, colonic tissue showed intact mucosal epithelia, ordered glands, clear structure, and no ulceration, as well as no inflammatory cell infiltration. In contrast, for rats in the WLC group, colonic tissues showed ulcers, damaged mucosa, disordered glandular structure, apparent edema in and underneath the mucosa, and infiltration of inflammatory cells. The above symptoms were gradually improved after moxibustion treatment, as shown in the three pictures on the right in Figure 3A. The 9M group showed the best improvement: epithelial mucosa and glands had an ordered structure, ulcer surface had new epithelial cells, and most of the infiltrating inflammatory cells disappeared. The condition of the colonic tissue in the 9M group was close to that in the BC group.

We further scrutinized the morphology of colonic mucosal epithelia by electron microscopy (Figure 3B). Our observations under electron microscopy were consistent with those of colonic tissue under light microscopy. For normal rats in the BC group, colonic mucosal epithelia showed intact microvilli in good order, tight junctions between cells, and clear mucous granules in the cytoplasm. On the contrary, colonic mucosal epithelia in the WLC group (without moxibustion treatment) contained damaged microvilli with uneven length, loose cell junctions, and unclear dissolved mucous granules in the cytoplasm. Symptoms of UC were greatly improved after moxibustion treatment. In particular, in the 9M group, colonic mucosal epithelia showed a similar condition to that in the rats without UC: relatively intact and ordered microvilli, tight cell junctions, and even cellular matrix.

Effect of moxibustion on secretion of IL-8 and IL-10

In order to determine whether the effectiveness of moxibustion treatment on inflammation of UC was linked to immunological abnormalities, we measured the levels of two inflammatory mediators, IL-8 and IL-10, which are genetically related to the immune system[20,21]. Serum levels of IL-8 and IL-10 in normal and UC rats are summa-

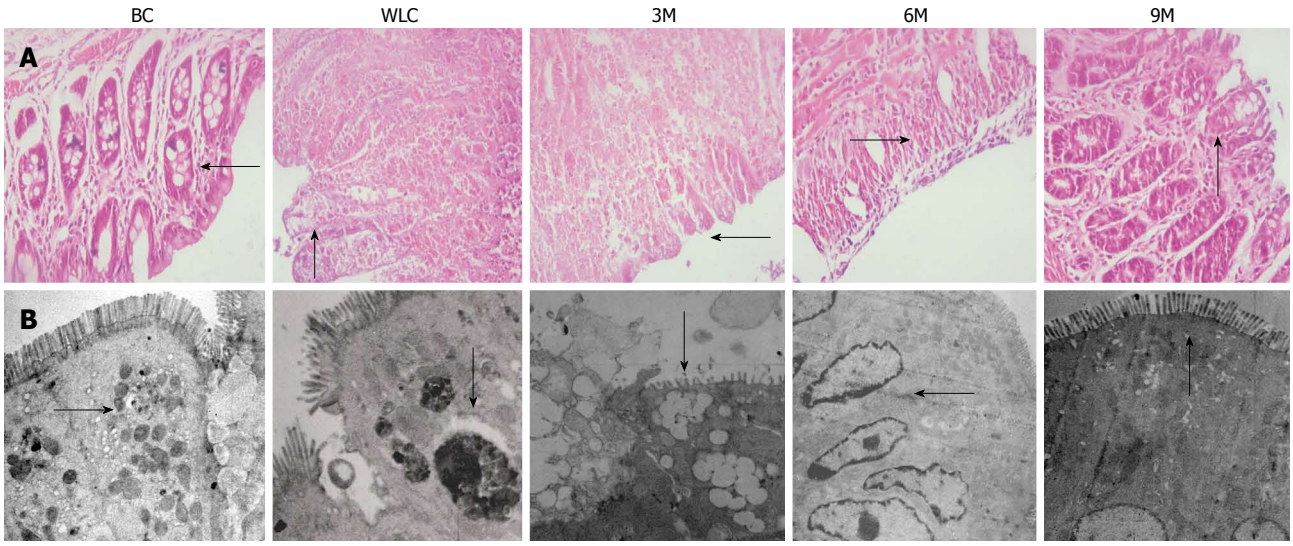
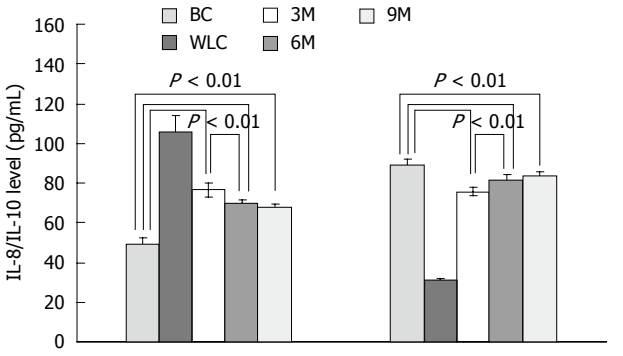


Figure 3 Histological features and morphology of colonic tissue. A: Histological analysis of colonic tissue sections stained with HE. The finer details of the epithelial cells can be seen clearly; B: Morphology of colonic mucosal epithelia observed by electron microscopy. From left to right in both A and B: Blank control (BC), waiting list control (WLC), 3-min moxibustion (3M), 6-min moxibustion (6M) and 9-min moxibustion (9M) groups. Treatment in the 9M group gave the best results, which were close to that of the normal rats without ulcerative colitis. Black arrow: Representative features of colonic tissue (magnification: A: 400 ×; B: 2000 ×).



	IL-8	IL-10
BC	48.99	89.34
WLC	105.46	30.83
3M	76.61	75.64
6M	69.78	80.90
9M	67.41	83.46

Figure 4 Interleukin-8 and interleukin-10 serum levels in normal rats (blank control), ulcerative colitis rats without (waiting list control) and with (3-min, 6-min and 9-min) moxibustion treatment. The measurements were done by ELISA. Moxibustion treatment increased secretion of interleukin (IL)-8 and inhibited that of IL-10. Significant differences in IL-8 and IL-10 of 3-min moxibustion (3M) *vs* blank control (BC), 6-min moxibustion (6M) *vs* BC, 9-min moxibustion (9M) *vs* BC, 3M *vs* waiting list control (WLC), 6M *vs* WLC, and 9M *vs* WLC were found with $P < 0.01$ for each comparison. For parallel comparisons, the differences in IL-8 and IL-10 levels were significant for 3M *vs* 6M ($P < 0.01$) and 3M *vs* 9M ($P < 0.01$). For better presentation, only partial comparisons are marked here. For 6M *vs* 9M, although the difference was no longer significant ($P > 0.05$), the tendency for IL-8 level to decrease and IL-10 level to increase was still noticeable.

rized and shown in Figure 4. IL-8 level in the WLC, 3M, 6M and 9M groups was significantly increased compared with that in the BC group ($P < 0.01$). In order to quantify the efficacy, we compared the IL-8 level among the different groups. The results in the 3M *vs* 6M and 3M *vs* 9M groups indicated significant differences in IL-8 ($P < 0.01$), and a tendency to decrease gradually to the normal level when UC rats received 9 min moxibustion treatment. The difference between the 6M and 9M groups was no longer significant ($P > 0.05$).

The results for IL-10 levels were similar to those for IL-8, except for the increasing/decreasing tendency. IL-10 level showed a tendency to increase to the normal level as the duration of moxibustion treatment increased, whereas IL-8 level had a tendency to decrease to the normal level. For the parallel comparison of 6M *vs* 9M, although the difference was no longer significant ($P > 0.05$), the tendency for the level of IL-10 to approach the normal level was still noticeable when the duration of treatment increased from 6 to 9 min. Due to the physical tolerance of UC rats, we reached 9 min of moxibustion treatment and did not increase the duration.

Effect of moxibustion on expression of TLR9 and NF-κB p65

The other two factors that play significant roles in immune responses, TLR9 and NF-κB p65, are also intrinsically associated with the inflammatory reaction of UC[22,23,29]. In order to investigate the intrinsic connection between effectiveness of moxibustion treatment in UC rats and expression of those two factors, we compared their expression among the different groups of normal and UC rats. Figures 5 show direct visualization of the differences of both TLR9 and NF-κB p65 expression in colonic tissue of normal rats (BC), moxibustion-free UC rats (WLC), and moxibustion-treated UC rats (3M, 6M and 9M).

We further summarized and compared such differences quantitatively, as shown in Figure 6. Expression of both TLR9 and NF-κB p65 in UC rats (WLC, 3M, 6M and 9M) showed a significant decrease ($P < 0.01$), compared to that in normal rats (BC). For UC rats, the differences of 3M *vs* WLC, 6M *vs* WLC, and 3M *vs* WLC were

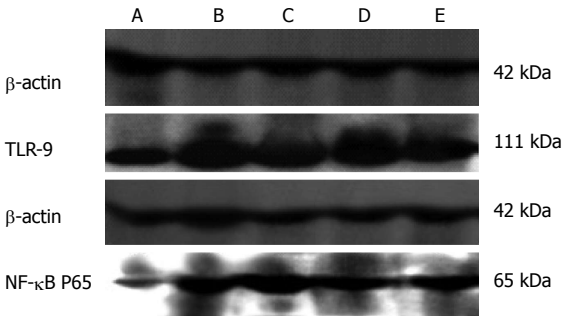
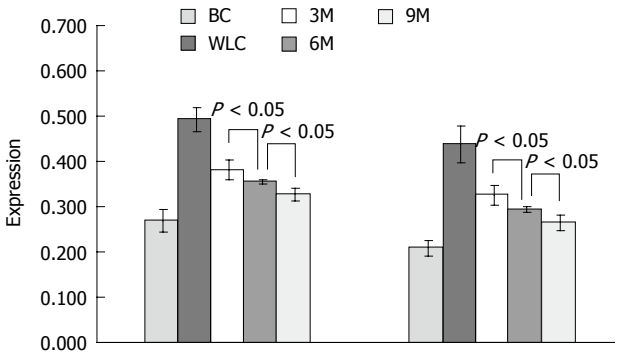


Figure 5 Expression of Toll-like receptor 9 and nuclear factor-κB p65 in colonic tissue of rats. A-E corresponds to the expression in colonic tissue of rats from blank control, waiting list control, 3-min, 6-min and 9-min moxibustion groups, respectively. Extracts of colonic tissues (approximately 25 μg) from normal or ulcerative colitis rats were assessed by sodium dodecyl sulphate polyacrylamide gel electrophoresis and were probed with Toll-like receptor (TLR) 9 as well as nuclear factor (NF)-κB p65 antibody. Antibody against β-actin was used as a loading control.



	TLR-9	NF-κB P65
BC	0.269	0.209
WLC	0.492	0.437
3M	0.380	0.326
6M	0.355	0.293
9M	0.327	0.265

Figure 6 Effect of moxibustion on expression of Toll-like receptor 9 as well as nuclear factor-κB p65. Expression of Toll-like receptor (TLR)9 as well as nuclear factor (NF)-κB p65 showed significant differences among all moxibustion-treated groups [3-min moxibustion (3M), 6-min moxibustion (6M) and 9-min moxibustion (9M)] and moxibustion-free groups [blank control (BC) and waiting list control (WLC)] with $P < 0.01$ for each comparison. For parallel comparisons between the moxibustion-treated groups, the differences were still significant for 3M *vs* 6M ($P < 0.05$), 3M *vs* 9M ($P < 0.05$), and 6M *vs* 9M ($P < 0.05$). The results for ulcerative colitis (UC) rats in the 9M group were the closest to the normal rats (BC). The results implied that 9 min moxibustion treatment resulted in the best improvement of UC within the physical tolerance of UC rats.

also significant ($P < 0.05$). The above results implied that moxibustion had an effect on expression of TLR9 and NF-κB p65 in UC rats. We further compared their expression in UC rats receiving different durations of moxibustion treatment. As the duration of moxibustion treatment in UC rats increased, the amount of TLR9 and NF-κB p65 expression decreased simultaneously. Significant differences ($P < 0.05$) still existed among all different comparisons (3M *vs* 6M, 3M *vs* 9M, and 6M *vs* 9M). The level of expression in UC rats that received 9 min moxibustion was the lowest, which was closest to the levels in normal rats without UC. On the contrary, the level of expression in UC rats without moxibustion treatment was the highest, which differed most from the level in normal rats. The above comparisons of TLR9 and NF-κB p65 among different groups regardless of moxibustion imply that moxibustion is an effective alternative treatment for UC in rats.

DISCUSSION

The significant decrease in DAI indicates that moxibustion is effective in improving the disease activity of UC rats. To be specific, moxibustion treatment can increase body mass, improve fecal viscosity, and reduce rectal bleeding. Our comparison of DAI among moxibustion-treated groups suggests that the efficacy of moxibustion depends on the duration of treatment, and 9 min treatment gave the best improvement of disease activity.

Our histological observations of colonic tissue and mucosal epithelia agree with the disease activity results: (1) without moxibustion treatment, ulcers, damaged mucosa, disordered glandular structure, edema, and infiltration of inflammatory cells appeared in the colonic tissue of UC rats; (2) damaged microvilli with uneven length, loose cell junctions, and unclear dissolved mucous granules in the cytoplasm were seen in colonic mucosal epithelia; (3) with moxibustion treatment, the above abnormalities showed significant improvements ($P < 0.05$); and (4) after 9 min treatment in each session, the features of colonic tissue and mucosal epithelia of UC rats showed the closest similarity to those of normal rats without UC.

UC is one type of nonspecific inflammatory bowel disease. A combination of genetic and environmental factors is widely accepted as the main cause of UC. The pathogenesis of UC is closely associated with immune and inflammatory responses. Inflammatory mediators IL-8, IL-10, TLR9 and NF-κB p65 are closely connected with immune and inflammatory responses[29-38].

IL-8 is a proinflammatory cytokine and a major chemoattractant and activator of neutrophils. IL-8 is closely connected to neutrophil recruitment, activation of the immune response, and promotion of inflammation. It activates immune responses through recruiting neutrophils to a site of inflammation as well as inducing neutrophils to bind to the extracellular matrix of cells, leading to inflammation[30,31]. IL-10 is an anti-inflammatory molecule that can suppress *in vitro* production of cytokines, and is involved in regulating immune and inflammatory responses[32]. Our comparison of the levels of IL-8/IL-10 among different groups suggest: (1) IL-8 level is correlated positively with severity of UC, whereas IL-10 level has a negative correlation; (2) moxibustion is effective in treating UC by significantly reducing IL-8 level while increasing IL-10 level; (3) within the physical tolerance limit of rats, a longer duration of moxibustion treatment achieves better efficacy; and (4) moxibustion treatment suppresses secretion of proinflammatory cytokine IL-8 while activating secretion of anti-inflammatory cytokine IL-10, thereby inhibiting the release of inflammatory cells and further interrupting the inflammatory response of UC.

In mammals, TLR9 plays a role in recognizing conserved pathogen-associated molecular patterns and further in inducing the inflammatory innate immune responses[33,34]. More specifically, TLR9 mediates CpG-DNA signaling involved in signal transduction pathways[37,38]. NF-κB p65, as a subunit of the NF-κB transcription complex, plays a crucial role in inflammatory and immune responses[29,35,36]. Activation of NF-κB p65 is the last step of the intracellular signaling pathway from the cell surface to the nucleus. Increased expression of NF-κB p65 has been reported in patients with UC[39]. By comparing the expression of TLR9 and NF-κB p65 in rat colonic tissue among different groups, several observations were made: (1) expression of NF-κB p65 in colonic tissue of UC rats was higher than in normal rats, indicating activation of NF-κB p65 in UC; (2) moxibustion treatment reduced expression of TLR9 and NF-κB p65 in UC; and (3) the degree of expression decreased significantly with duration of treatment, and 9 min treatment yielded the best results. In addition to its effectiveness, our results suggest that moxibustion treatment inhibits activation of NF-κB p65, thereby blocking the signal transduction pathways of TLR9.

In summary, in this study, we investigated the effectiveness of moxibustion treatment in UC rats, and quantitatively assessed the efficacy from a modern medicine perspective. In addition, we established the relation between moxibustion therapy and immune or inflammatory responses by observing IL-8, IL-10, TLR9 and NF-κB p65 expression. Our results indicate that moxibustion treatment improves disease activity, repairs damaged colonic mucosa, suppresses secretion of serum IL-8 while activating that of IL-10, inhibits activation of NF-κB p65, and decreases expression of TLR-9 in UC rats. We propose that the effect of moxibustion therapy in UC rats is probably related to inhibition of inflammatory cells by suppressing secretion of proinflammatory cytokine IL-8 and activating secretion of anti-inflammatory cytokine IL-10, and blocking of the inflammatory signaling transduction pathway by inhibiting activation of transcription factor NF-κB p65 and repressing the pattern-recognition receptor TLR9. Our results provide direct evidence of why moxibustion is effective in the treatment of UC from a modern medicine perspective.

COMMENTS

Background

Moxibustion (application of heat to acupuncture points), as an alternative and complementary medicine, has been widely used for treating inflammatory bowel disease, due to its relatively low level of clinical side effects and higher compliance in patients compared to drug therapy or surgical procedures. Ulcerative colitis (UC) is a nonspecific inflammatory bowel disease that afflicts millions of people worldwide. Many studies have reported that moxibustion is effective in UC. Nevertheless, there is little or insufficient information for evaluation of the effectiveness and therapeutic mechanism of moxibustion.

Research frontiers

The combination of genetic and environmental factors is widely accepted as the cause of UC. In the area of moxibustion treatment of UC, the research hotspot is to establish whether moxibustion is effective, and only rarely do studies focus on the underlying mechanisms of its therapeutic effects.

Innovations and breakthroughs

The authors quantitatively investigated the efficacy of moxibustion in the treatment of UC rats by observing the changes in physical and biological parameters related to the immune system: disease activity index, colonic epithelial glandular arrangement, interleukin (IL)-8, IL-10, Toll-like receptor (TLR)9, and nuclear factor (NF)-κB p65. The results indicate that moxibustion improves disease activity of UC rats, repairs damaged colonic mucosa, suppresses secretion of serum IL-8 while activating that of IL-10, inhibits activation of NF-κB p65, and decreases expression of TLR-9 in UC rats. They also propose a connection between the therapeutic efficacy of moxibustion therapy effect and inflammatory responses. The results provide direct scientific evidence of why moxibustion is effective in the treatment of UC.

Applications

This study provides quantitative information about the efficacy of moxibustion for future clinical practice. The efforts to probe into the comprehensive theoretical system of moxibustion treatment for UC offer direct scientific evidence of its therapeutic effects from a modern medicine perspective.

Terminology

Moxibustion is a traditional Chinese medicine technique that involves burning of mugwort (a small spongy herb) to facilitate healing, strengthen the blood, stimulate the flow of qi, and maintain general health. UC is one of the most common types of inflammatory bowel disease. UC affects the colon and rectum. The pathogenesis of UC mainly involves erosion and ulceration, with common clinical manifestations of diarrhea, weight loss, abdominal pain, bloody stools, fever, and fatigue.

Peer review

This was an interesting study about the roles of moxibustion in regulating IL-8, IL-10, TLR9 and NF-κB p65 in UC. The paper is well written.

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P- Reviewer: Beissner F **S- Editor**: Gou SX **L- Editor**: Logan S **E- Editor**: Zhang DN

World Journal of Gastroenterology

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DOI: 10.3748/wjg.v20.i32.11305

World J Gastroenterol 2014 August 28; 20(32): 11305-11312
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

RESEARCH REPORT

Role of the ^{13}C-methacetin breath test in the assessment of acute liver injury in a rat model

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Supported by Beijing Health System Advanced Health Technology Talent Cultivation Plan, No. 2011-2-08
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Received: June 8, 2014 Revised: July 18, 2014
Accepted: July 29, 2014
Published online: August 28, 2014

Abstract

AIM: To evaluate the role of the ^{13}C-methacetin breath test (^{13}C-MBT) in the assessment of acute liver injury in a rat model.

METHODS: Acute liver injury in rats was induced by a single intraperitoneal injection of D-galactosamine (D-GalN). Forty-eight male Sprague-Dawley rats were randomly assigned to a control group (n = 8) and five model groups (each n = 8), and acute liver injury was assessed at different time points (6, 12, 24, 48 and 72 h) after D-GalN injection. The ^{13}C-MBT, biochemical tests, 15-min retention rate of indocyanine green (ICG_{R15}), and liver biopsy were performed and compared between the control and model groups. Correlations between parameters of the ^{13}C-MBT (T_{max}, MV_{max}, CUM_{120} and DOB_{max}), biochemical tests, ICG_{R15} and liver necrosis score were also analyzed using Spearman's correlation analysis.

RESULTS: T_{max}, MV_{max}, CUM_{120} and DOB_{max}, as well as most of the traditional methods, correlated with the liver necrosis score (r = 0.493, P < 0.05; r = -0.731, P < 0.01; r = -0.618, P < 0.01; r = -0.592, P < 0.01, respectively). MV_{max}, CUM_{120} and DOB_{max} rapidly decreased and were lower than those in the controls as early as 6 h after D-GalN injection (3.84 ± 0.84 *vs* 5.06 ± 0.78, P < 0.01; 3.35 ± 0.72 *vs* 4.21 ± 1.44, P < 0.05; 52.3 ± 20.58 *vs* 75.1 ± 9.57, P < 0.05, respectively) and reached the lowest point 24 h after D-GalN injection. MV_{max}, CUM_{120} and DOB_{max} returned to normal levels 72 h after D-GalN injection and preceded most of the traditional methods, including liver biopsy.

CONCLUSION: The ^{13}C-MBT is a sensitive tool for the timely detection of acute liver injury and early prediction of recovery in a rat model. Further clinical studies are warranted to validate its role in patients with acute liver injury.

Key words: ^{13}C-methacetin breath test; Acute liver injury; Liver function; Animal model

Core tip: The ^{13}C-methacetin breath test (^{13}C-MBT) is a promising tool for the assessment of metabolic liver function. Previous studies have mainly been conducted in the setting of chronic liver disease; however, evidence on acute liver injury is scanty. The present study evaluated the role of ^{13}C-MBT in the assessment of acute liver injury in an animal model and showed that ^{13}C-MBT was sensitive for the timely detection of acute liver injury and for early prediction of liver function recovery.

Zhu D, Zhang H, Mao JY, Wang HY, Li X, Xu YQ. Role of the ^{13}C-methacetin breath test in the assessment of acute liver injury

in a rat model. *World J Gastroenterol* 2014; 20(32): 11305-11312 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11305.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11305

INTRODUCTION

Acute liver disease may progress to fulminant hepatic failure, as a result of which, a high proportion of patients will die unless liver transplantation is performed[1]. Therefore, timely and accurate assessment of the degree of liver damage in acute liver disease outweighs specific disease etiologies with regard to treatment and prognosis[2]. However, current methods of disease severity assessment in patients with acute liver disease are far from optimal. Conventional biochemical tests, such as transaminase, bilirubin, albumin (ALB) plasma levels and prothrombin time (PT), provide information on a mixture of injury and function, but none of these are regarded as a sensitive and reliable marker of the severity of liver injury[3]. Although the 15-min retention rate of indocyanine green (ICG_{R15}) is considered to be a reliable method for assessing liver function impairment[4-6], several drawbacks, such as cumbersome processing, potential allergic reaction, and its invasive nature limit its use in clinical settings. Many patients with liver disease, acute or chronic, undergo a liver biopsy to guide therapeutic decisions[7]. However, liver biopsy is highly invasive and impractical as a follow-up test. Thus, a simple, noninvasive and powerful alternative to liver biopsy in the management of liver disease is required.

Non-invasive breath tests using carbon isotope ^{13}C have been proposed as a measure of metabolic liver function. ^{13}C can be incorporated into organic substances and metabolized by the liver. The by-product of this metabolism is $^{13}CO_2$, which changes the $^{13}CO_2/^{12}CO_2$ isotope ratio of the patient's exhaled breath. Measuring the $^{13}CO_2/^{12}CO_2$ ratio in exhaled air enables quantification of microsomal function[8]. Thus, the breath test can be used early to evaluate quantitatively liver function damage or hepatic reserve function at hepatocyte metabolic level. The substances used in breath tests include aminopyrine, methacetin, caffeine, galactose and others[9]. Methacetin is widely used for breath tests because of its low toxicity in small doses. The ^{13}C-methacetin breath test (^{13}C-MBT) is considered a safe, simple and repeatable method for monitoring dynamic liver function.

The ^{13}C-MBT could accurately identify liver fibrosis and differentiate the grades of fibrosis in patients with chronic hepatitis C infection[7,8,10]. In patients with acute liver disease, preliminary evidence indicated that clinical improvement and normalization of biochemical parameters were accompanied by progressive improvement of the ^{13}C-MBT scores[11]. Detection of improvement using ^{13}C-MBT occurred 1-3 d earlier than any of the other clinical and laboratory parameters, suggesting that the ^{13}C-MBT might serve as a more sensitive decision-making tool for the follow-up of these patients in the setting of severe acute liver disease[12].

The present study was designed to preliminarily evaluate the role of the ^{13}C-MBT in the assessment of acute liver injury in an animal model. Parameters of the ^{13}C-MBT, as well as ICG_{R15} and biochemical variables, were tested and compared between the control and model groups (at different time points during the study); their associations with liver biopsy were also analyzed.

MATERIALS AND METHODS

Animals and materials

Forty-eight male Sprague-Dawley (SD) rats of similar age and weight (200 ± 20 g) were obtained from Shanghai Laboratory Animal Center and were fed a Purina laboratory chow, *ad libitum*, in an internal animal breeding house at Beijing Tiantan Hospital. The rats were individually housed in a temperature and humidity controlled environment with a 12-h light-dark cycle and were free to drink tap water until the week before the study began. All animals received human care in compliance with the "Guide for the Care and Use of Laboratory Animals" published by the National Academy Press[13]. The study was approved by local ethic committee of Beijing Tiantan Hospital, Capital Medical University. D-Galactosamine (D-GalN) was purchased from Sigma Chemical Company (St. Louis, MO, United States). Indocyanine green was purchased from Dandong Yichuang Pharmaceutical Co., Ltd. (Dandong, China). ^{13}C-methacetin powder and the Infra-Red Isotope Analyzer (IRIS) were purchased from WAGNER Analysen Technik (Bremen, Germany). A UV-vis spectrophotometer was purchased from Beijing Purkinje General Instrument Co., Ltd. (Beijing, China).

Experimental design

The 48 rats were randomly assigned to six groups, including a control group and five acute liver injury model groups (M_{6h}, M_{12h}, M_{24h}, M_{48h} and M_{72h}). The variables in the subscript after the capital letter "M" denote the time points after intraperitoneal injection. Acute liver injury was induced by a single intraperitoneal injection of D-GalN (450 mg/kg), which was dissolved in saline (0.6 mL/100 g of body weight)[14]. The rats in the control group received the same volume of saline as a substitute for D-GalN. ICG_{R15} and biochemical analyses were conducted at predetermined time points (6, 12, 24, 48 and 72 h) for each model group after D-GalN injection, while the ^{13}C-MBT was conducted 2 h ahead of the predetermined time point because of the time taken to perform the test. The rats were then sacrificed under anesthesia and liver tissues were harvested and used for histopathological analysis. The rats in the control group underwent the same procedure within 6 h after intraperitoneal injection of saline.

^{13}C-MBT

^{13}C-methacetin powder was completely dissolved in dis-

tilled water to a final concentration of 4 g/L. According to the experimental design, each rat was given ^{13}C-methacetin (200 mg/100 g of body weight) by gavage after 10 h fasting. For the ^{13}C-MBT, a home-made device was used to collect expiratory air; Shirin *et al*[9] describe the details of the device structure. Breath samples for $^{13}CO_2$ measurement were collected for 15 s continuously by an aspirator pump and were collected at baseline and 10, 20, 30, 40, 50, 60, 80, 100 and 120 min after gavage. Each sample was then transferred into a special aluminum covered plastic bag (100 mL capacity). The bag was closed with a plastic cork immediately at the end of exhalation and prepared for analysis. After connecting the bag to the IRIS, the machine analyzed the breath samples automatically and provided several valuable parameters such as DOB_{max}, T_{max}, MV_{max} and CUM_{120}. DOB_{max} is the peak value of the delta over baseline (DOB) curve, which reflects the change in the natural $^{13}CO_2/^{12}CO_2$ ratio exhaled to the ingestion of a labeled substrate during the test period. T_{max} reflects the time at which the peak amplitude of DOB curve appears. MV_{max} reflects the largest metabolic rate of $^{13}CO_2$, and CUM_{120} reflects cumulative percentage of administered dose of $^{13}CO_2$ recovered over time at 120 min[15,16].

ICG_{R15} and biochemical analyses

The ICG standard curve was drawn according to the manufacturer's instructions. Indocyanine green was completely dissolved in distilled water and prepared to a final concentration of 0.1 mg/mL. According to the experimental design, each rat was given a caudal intravenous injection of indocyanine green at 50 mg/100 g of body weight. Abdominal aortic puncture was performed to collect blood in a heparinized tube at 15 min after injection of ICG, followed by isolation of plasma with centrifugation at 1500 × *g* for 15 min. Plasma (0.25 mL) was extracted and diluted with 1.25 mL saline. Absorbance value of the plasma sample at 805 nm was determined by A UV-vis spectrophotometry determined the absorbance of the plasma sample at 805 nm, which were compared with the value for normal plasma. The standard curve was used to calculate the serum concentration of ICG. An automatic biochemical analyzer detected the biochemical parameters, including alanine transaminase (ALT), total bilirubin (TBIL), ALB and PT, from a further 0.6-1.0 mL of extracted plasma.

Hepatic histopathology

Rats were sacrificed after the ^{13}C-MBT, ICG_{R15} and blood collection were completed. Their livers were harvested and midsections of the left lobes were fixed with 10% formalin solution and processed for staining with hematoxylin and eosin (HE) and then studied by light microscopy. The extent of liver necrosis was determined by counting the number of necrotic foci under low-power field (LPF) magnification, using a × 10 objective. A necrotic area of 3600 μm^2 measured by a 0.01 mm microscope micrometer was defined as a necrotic focus. Ten LPFs were chosen randomly along with a W-shaped sampling path for each slice. The liver necrosis score was obtained by counting the total number of necrotic foci in 10 LPFs.

Statistical analysis

Data analysis was performed using SPSS 17.0 statistical analysis software (SPSS, Chicago, IL, United States). Continuous variables were described as mean ± SD and were analyzed by the Student's two-independent-sample *t* test (normal distribution) or Mann-Whitney non-parametric *U* test (skewed distribution). Normality of distribution was determined using the Kolmogorov-Smirnov test (cut-off at $P = 0.01$). Associations between the liver necrosis score and biochemical parameters, ICG_{R15} and the ^{13}C-MBT were described using Spearman's correlation analysis. All tests were two-sided and considered significant at $P < 0.05$.

RESULTS

Histopathological assessment of acute liver injury

In the control group, the hepatic architecture was normal, with little necrosis and inflammatory cell infiltration. At 6 h after intraperitoneal injection of D-GalN, the structure of the hepatic lobules was largely normal with spotty, focal necrosis and little inflammatory cell infiltration. At 12 h, the structure of hepatic lobules was slightly damaged, with focal necrosis and moderate inflammatory cell infiltration. At 24 h, there was an extensive area of central-central and central-portal bridging necrosis, containing many red cells trapped in the collapsed reticulin network, and significant inflammatory cell infiltration was observed. At 48 h, hepatic necrosis and inflammatory cell infiltration gradually reduced and at 72 h, hepatic lobular structure had returned to normal with very little inflammatory cell infiltration (Figure 1). Thus, the severity of liver necrosis increased with time after D-GalN injection, and reached a peak between 24 and 48 h, and then gradually returned to normal. The liver necrosis scores in the control group and model groups at 6, 12, 24, 48 and 72 h were 0.4 ± 0.2, 28.6 ± 18.5, 59.5 ± 9.5, 80.3 ± 4.2, 36.0 ± 3.4, and 21.1 ± 4.1, respectively. The liver necrosis scores in the model groups at 6, 12, 24, 48 and 72 h were significantly higher than those in the control group (Table 1).

Comparison of parameters of the ^{13}C-MBT between the model groups and control group

The mean value of T_{max} (min) in the control group and model groups at 6, 12, 24, 48 and 72 h were 18.3 ± 4.1, 21.7 ± 4.7, 23.3 ± 5.16, 33.3 ± 4.3, 28.3 ± 3.83, and 23.3 ± 5.16, respectively. Compared with the control group, the T_{max} in the model groups at 12 h, 24 h, 48 h and 72 h were significantly higher. The MV_{max} (%), CUM_{120} (%) and DOB_{max} (%) in the control group were 5.06 ± 0.78, 4.21 ± 1.44, and 75.1 ± 9.57, respectively. These three parameters followed a similar pattern and decreased simultaneously, reaching the lowest value at 24 h, and then returning to normal. MV_{max}, CUM_{120} and DOB_{max} in the model groups at 6, 12, 24 and 48 h were significantly

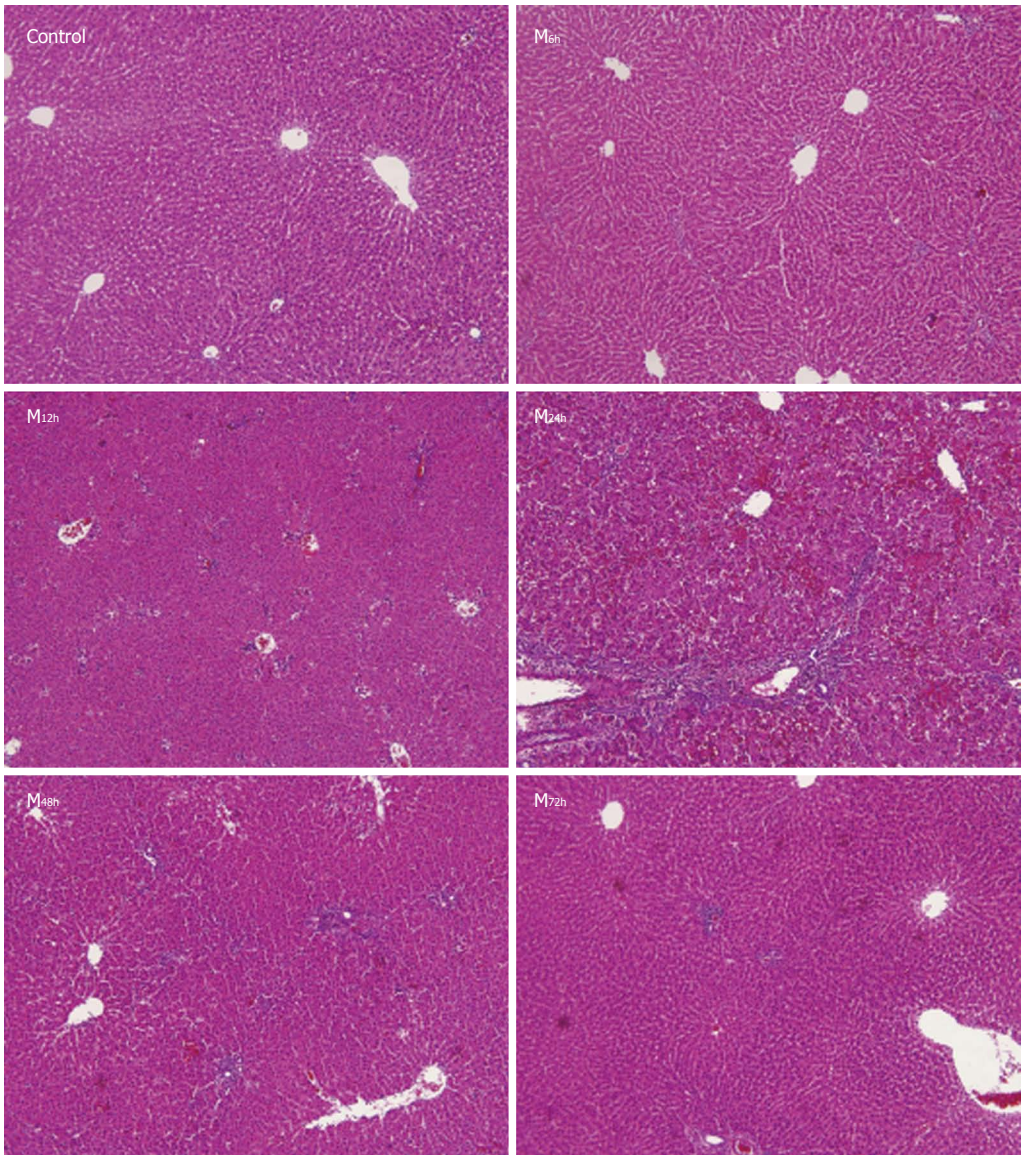

Figure 1 Histopathological findings of liver tissues in control and model groups (hematoxylin and eosin × 100). Control: Hepatic architecture was normal with little necrosis and inflammatory cell infiltration; M_{6h}: The structure of the hepatic lobules was largely normal with spotty, focal necrosis and little inflammatory cell infiltration; M_{12h}: The structure of hepatic lobules was slightly damaged, with focal necrosis and little inflammatory cell infiltration; M_{24h}: There was an extensive area of central-central and central-portal bridging necrosis containing many red cells trapped in the collapsed reticulin network, and significant inflammatory cell infiltration was observed; M_{48h}: Hepatic necrosis and inflammatory cell infiltration gradually reduced; M_{72h}: Hepatic lobular structure returned to normal, with very little inflammatory cell infiltration.

lower than those in the control group. At 72 h, the differences in these three parameters between the control group and model groups were no longer significant (Table 1, Figure 2). Correlation analysis of the parameters of the ^{13}C-MBT and liver necrosis score revealed that T_{max} was positively correlated with liver necrosis score (r = 0.493, $P < 0.05$), while MV_{max}, CUM_{120} and DOB_{max} were negatively correlated with liver necrosis score (r = -0.731, $P < 0.01$; r = -0.618, $P < 0.01$; r = -0.592, $P < 0.01$, respectively) (Table 1).

Comparison of biochemical blood tests and ICG_{R15} between the model groups and control group

The mean level of ALT in the control group was 45.5 ± 7.0 U/L. After injection of D-GalN, ALT level increased rapidly and reached a peak (994.3 ± 427.5 U/L) at 24 h, and then gradually decreased. ALT levels in the model groups at 12, 24 and 48 h were significantly higher than that in the control group (444.1 ± 230.4 U/L, $P < 0.01$; 994.3 ± 427.5 U/L, $P < 0.01$; 436.8 ± 103.7 U/L, $P < 0.01$, respectively). Correlation analysis revealed that ALT was positively correlated with liver necrosis score (r = 0.768, $P < 0.01$). The changes in TBIL following D-GalN injection were similar to those of ALT. Levels of TBIL (μmol/L) in the model groups at 12, 24, 48 and 72 h were significantly higher than that in the control group (2.41 ± 0.98, $P < 0.05$; 13.74 ± 0.82, $P < 0.001$; 12.99 ± 1.67, $P < 0.001$; 12.81 ± 0.71, $P < 0.001$, respectively). Correlation analysis revealed that TBIL was positively correlated with liver necrosis score (r = 0.368,

Table 1 Comparison of parameters of the ^{13}C-methacetin breath test and liver necrosis score between the control and model groups

Group	T_{max} (min)	MV_{max}	CUM_{120}	DOB_{max}	Liver necrosis score
Control	18.3 ± 4.1	5.06% ± 0.78%	4.21% ± 1.44%	75.1% ± 9.57%	0.4 ± 0.2
M_{6h}	21.7 ± 4.7	3.84% ± 0.84%^{b}	3.35% ± 0.72%^{a}	52.3% ± 20.58%^{a}	28.6 ± 18.5^{b}
M_{12h}	23.3 ± 5.2^{a}	3.52% ± 1.32%^{b}	2.77% ± 1.40%^{b}	40.1% ± 4.13%^{b}	59.5 ± 9.5^{b}
M_{24h}	33.3 ± 4.3^{b}	2.55% ± 1.31%^{b}	2.67% ± 0.71%^{b}	37.3% ± 18.50%^{b}	80.3 ± 4.2^{b}
M_{48h}	28.3 ± 3.8^{a}	2.72% ± 0.31%^{b}	2.77% ± 0.81%^{b}	52.23% ± 14.16%^{a}	36.0 ± 3.4^{b}
M_{72h}	23.3 ± 5.2^{a}	4.72% ± 0.81%	4.12% ± 0.44%	69.1% ± 13.96%	21.1 ± 4.1^{a}

DOB_{max} is the peak value of the delta over baseline (DOB) curve, which reflects the change in the natural $^{13}CO_2/^{12}CO_2$ ratio exhaled to the ingestion of a labeled substrate during the test period. T_{max} reflects the time at which the peak amplitude of DOB curve appears. MV_{max} reflects the largest metabolic rate of $^{13}CO_2$, and CUM_{120} reflects the cumulative percentage of administered dose of $^{13}CO_2$ recovered over time at 120 min. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control.

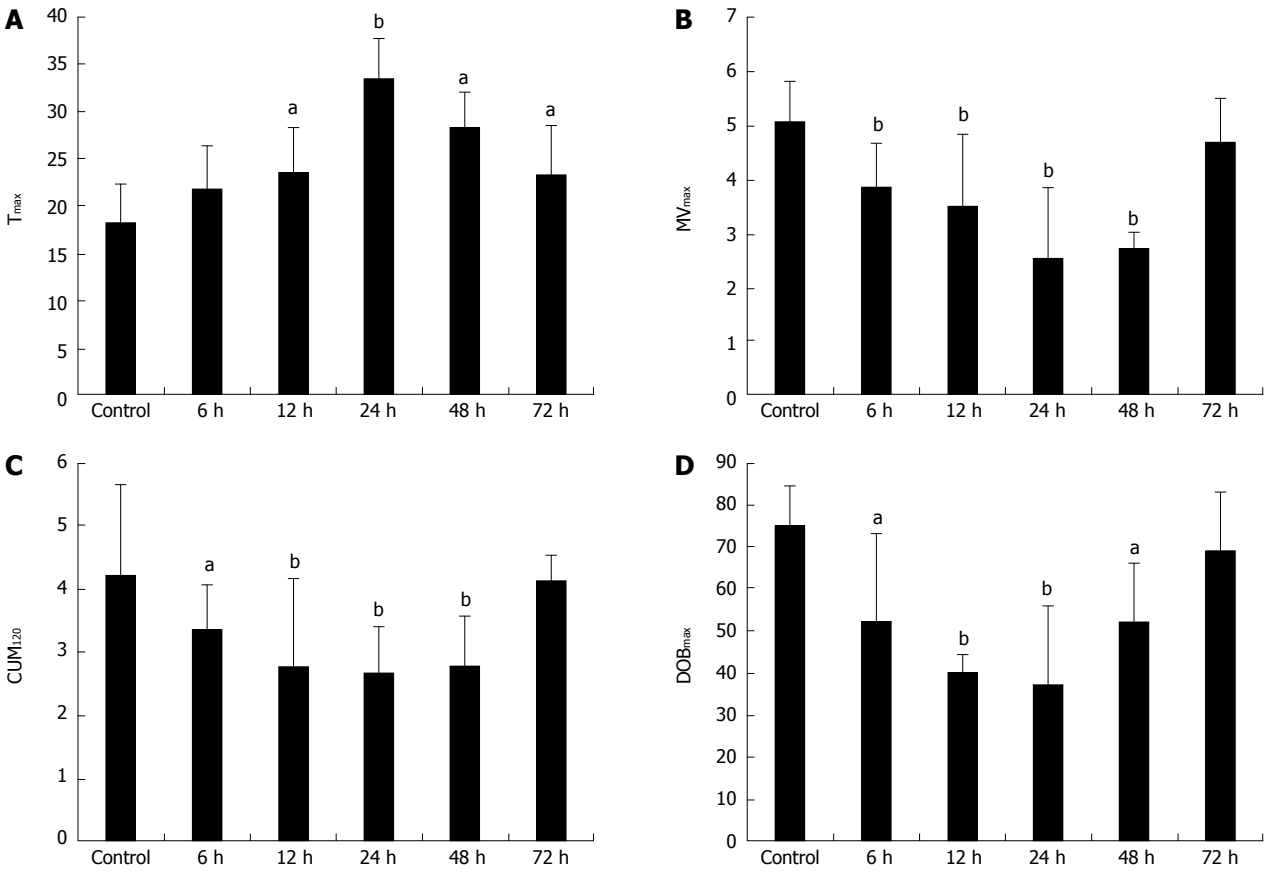

Figure 2 Comparison of parameters of ^{13}C-methacetin breath test between control and model groups at different time points after D-GalN injection. A: T_{max} (min); B: MV_{max} (%); C: CUM_{120} (%); D: DOB_{max} (%). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control.

$P = 0.01$). The mean level of ALB in the control group was 34.52 ± 2.69 g/L. After injection of D-GalN, ALB level decreased and remained low at 72 h. ALB levels (g/L) in the model groups at 24, 48 and 72 h were significantly lower than that in the control group (32.08 ± 4.04, $P < 0.05$; 31.48 ± 1.57, $P < 0.05$; 28.79 ± 1.73, $P < 0.001$, respectively). Correlation analysis revealed that ALB was not correlated with liver necrosis score ($r = 0.009$, $P > 0.05$). PT values in the model groups at each time point were significantly longer than that in the control group ($P < 0.01$), and correlation analysis revealed that PT was positively correlated with liver necrosis score ($r = 0.627$, $P < 0.01$) (Table 2). ICG_{R15} (%) in the control group and model groups at 6, 12, 24, 48 and 72 h were 2.8 ± 0.4, 3.1 ± 0.6, 6.8 ± 1.4, 17.0 ± 2.3, 11.5 ± 1.6 and 5.7 ± 1.2, respectively. Compared with the control group, ICG_{R15} in the model groups at 12, 24 and 48 h were significantly higher ($P < 0.05$; $P < 0.001$; $P < 0.001$, respectively). Correlation analysis revealed that ICG_{R15} was positively correlated with liver necrosis score ($r = 0.604$, $P < 0.01$) (Table 2).

DISCUSSION

In recent years, the role of the ^{13}C-MBT to assess liver function has been investigated in patients with chronic liver disease. Matsumoto *et al*[11] found that the ^{13}C-MBT value (the ^{13}C recovery over 30 min) was significantly re-

Table 2 Comparison of biochemical tests and 15-min retention rate of indocyanine green between control group and model groups

Group	ALT (U/L)	TBIL (μmol/L)	PT (s)	ALB (g/L)	ICG15
Control	45.5 ± 7.0	1.36 ± 0.61	13.55 ± 0.55	34.52 ± 2.69	2.8% ± 0.4%
M_{6h}	166.8 ± 40.7	1.56 ± 0.72	18.89 ± 3.38^b	33.93 ± 1.27	3.1% ± 0.6%
M_{12h}	444.1 ± 230.4^b	2.41 ± 0.98^a	23.99 ± 3.09^b	33.65 ± 1.57	6.8% ± 1.4%^{a}
M_{24h}	994.3 ± 427.5^b	13.74 ± 0.82^b	36.36 ± 2.45^b	32.08 ± 4.04^a	17.0% ± 2.3%^{b}
M_{48h}	436.8 ± 103.7^b	12.99 ± 1.67^b	33.05 ± 3.22^b	31.48 ± 1.57^a	11.5% ± 1.6%^{b}
M_{72h}	238.7 ± 70.7	12.81 ± 0.71^b	29.13 ± 2.94^b	28.79 ± 1.73^b	5.7% ± 1.2%

$^aP < 0.05$, $^bP < 0.01$ *vs* control. ALB: Albumin; ALT: Alanine transaminase; AST: Aspartate transaminase; ICG_{R15}: The 15-min retention rate of indocyanine green; PT: Prothrombin time; TBIL: Total bilirubin.

duced in patients with chronic aggressive hepatitis and in those with liver cirrhosis, but not in patients with chronic persistent hepatitis or healthy controls. Patients with either advanced cirrhosis or hepatocellular carcinoma showed significantly lower values than those with well-compensated cirrhosis, indicating that the ^{13}C-MBT[11] can effectively evaluate the severity of liver damage. Goetze *et al*[10] tested 100 patients with untreated chronic HCV infection, and 100 age- and sex-matched healthy volunteers using the ^{13}C-MBT following ingestion of 75 mg methacetin. They found that the ^{13}C-MBT was an accurate tool for measuring the degree of inflammation and fibrosis in patients with chronic HCV infection and normal serum alanine aminotransferase (NALT)[10]. Further studies have supported the role of the ^{13}C-MBT in the assessment of chronic liver disease[17-19]. A few studies have focused on the assessment of the degree of hepatic damage in acute liver disease. A recent study showed that the ^{13}C-MBT was a sensitive test and may be a useful tool to evaluate functional liver mass in animal models of acute liver failure and cirrhosis[9].

The present study was designed to explore the role of the ^{13}C-MBT in assessing acute liver injury using a rat model. The histopathological analysis showed that the severity of liver necrosis increased with time after intraperitoneal injection of D-GalN, reached a peak between 24 and 48 h, and then gradually returned to normal. Correlation analysis revealed that parameters of the ^{13}C-MBT, as well as most of the traditional methods, correlated with liver necrosis score, which was in accordance with the morphologic findings. The changes in MV_{max}, CUM_{120} and DOB_{max} reflected the severity of liver injury after D-GalN injection. Liver function abnormalities were recognized by the ^{13}C-MBT as early as 6 h after initiation of liver damage, which was earlier than most of the traditional methods, including ALT, TBIL, ALB, ICG_{R15} and liver biopsy. In addition, the ^{13}C-MBT reflected the recovery of liver injury after D-GalN injection. The parameters of the ^{13}C-MBT gradually returned to baseline levels after 24 h, which was in accordance with the liver necrosis score. The differences in the parameters of the ^{13}C-MBT between the model groups and control group at 72 h were no longer significant, while most of the other methods, including liver histopathology are not sufficiently informative, indicating that the ^{13}C-MBT may be a sensitive tool for predicting recovery from acute liver injury.

Some limitations in the present study should be acknowledged. Firstly, the limited number of rats in each group may have lowered the statistical power and led to false positive or false negative results. This could be minimized by enlarging the sample size in future studies. Secondly, we used a self-defined liver necrosis score to evaluate the severity of liver injury, according to previously published literature, as no consensus standardized method or scoring system is available in the setting of acute liver injury[20]. We found that the liver necrosis score reflected the severity of liver injury following corroboration of the results with other methods. Thirdly, whether the results of animal experiments are applicable to humans is unknown, and further studies on patients to validate the results of our study are warranted. Lastly, we used liver biopsy as the standard method to evaluate the role of the ^{13}C-MBT in acute liver injury. Although liver biopsy has been widely regarded as the "gold standard" for defining liver disease status, it also has drawbacks that have prompted questions about its value[21,22]. However, there are currently no other tools that can serve as alternative methods. Although the Child-Turcotte-Pugh score and the model for end-stage liver disease score were reported to be valuable to predict liver disease progression[23-25], their suitability for use in animal models is unknown.

In conclusion, the results of the present study suggest that the ^{13}C-MBT may be a valuable tool to assess liver function in acute liver injury in a rat model. The ^{13}C-MBT is sensitive for timely detection of acute liver injury and early prediction of liver function recovery. To validate its role in patients, further clinical studies are warranted.

ACKNOWLEDGMENTS

We thank David B Nwinee from International School of Capital Medical University for his help with the manuscript.

COMMENTS

Background

The ^{13}C-methacetin breath test (^{13}C-MBT) has been proposed as a promising tool for the assessment of metabolic liver function in the setting of chronic liver disease. However, evidence of its role in acute liver injury is scanty and anecdotal.

Research frontiers

Recent studies have shown that the ^{13}C-MBT is an accurate tool to identify liver fibrosis and differentiating grades of fibrosis in patients with chronic hepatitis C infection. In patients with acute liver disease, preliminary evidence indicated that clinical improvement and normalization of biochemical parameters were accompanied by progressive improvements in ^{13}C-MBT scores. The ^{13}C-MBT detected improvement 1-3 d earlier than the other clinical and laboratory parameters, suggesting that the ^{13}C-MBT might serve as a more sensitive decision-making tool for the follow-up of patients with severe acute liver disease.

Innovations and breakthroughs

Although the ^{13}C-MBT has been studied in the clinical setting for several years, most of these studies were case reports or case series, and were related to chronic liver disease. There are few studies focusing on patients with acute liver injury, probably because of concerns for the safety of patients with serious conditions. This study evaluated the role of the ^{13}C-MBT in the assessment of acute liver injury in an animal model. Parameters of the ^{13}C-MBT, as well as ICG_{R15} and biochemical variables, were tested and compared between the control and model groups (at different time points during the study); their associations with liver biopsy were also analyzed.

Applications

The ^{13}C-MBT is a sensitive tool for timely detection of liver injury and early prediction of liver function recovery in the setting of acute liver disease.

Terminology

^{13}C-MBT: ^{13}C can be incorporated into organic substances and metabolized by the liver. The by-product of this metabolism is $^{13}CO_2$, which changes the $^{13}CO_2/^{12}CO_2$ isotope ratio of the patient's exhaled breath. Measuring the $^{13}CO_2/^{12}CO_2$ ratio in exhaled air enables quantification of microsomal function. Methacetin is widely used for breath tests because of its low toxicity in small doses.

Peer review

Zhu *et al* presented the results of their study on the role of ^{13}C-MBT in the assessment of acute liver injury induced by *D*-galactoamine in rats. It has to be noted that there are already some studies concerning the role of ^{13}C-MBT in the evaluation of hepatic damage/reserve during both chronic and acute liver damage. However, to date, the ^{13}C-MBT has not been evaluated significantly or comprehensively in the acute setting. Overall, the study is well-designed, results are somewhat interesting and the paper is well-written.

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P- Reviewer: Vespasiani-Gentilucci U **S- Editor**: Qi Y
L- Editor: Stewart G **E- Editor**: Zhang DN

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i32.11313

World J Gastroenterol 2014 August 28; 20(32): 11313-11320
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE CONTROL STUDY

Lower serum folate is associated with development and invasiveness of gastric cancer

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Supported by National Science Council, Executive Yuan, No. NSC-96-2314-B-075A-007, No. NSC100-2628-B005-002MY4, No. NSC101-2320-B-005-006-MY3 and No. NSC 101-2911-I-005-301; and the ATU plan of the Ministry of Education, Taiwan
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Received: January 23, 2014 Revised: March 26, 2014
Accepted: April 30, 2014
Published online: August 28, 2014

Abstract

AIM: To evaluate the associations of serum folate level with development, invasiveness and patient survival of gastric cancer.

METHODS: In this nested case-control study, patients with newly diagnosed gastric cancer undergoing gastrectomy were enrolled, and patients receiving chemotherapy prior to surgery, with other concurrent malignancy, or of the aboriginal and alien populations were excluded. In total, 155 gastric cancer patients and 149 healthy controls were enrolled for determination of serum folate levels and their correlation with gastric cancer. Using the median value of serum folate computed among the overall population as the cutoff value, the associations between serum folate and gastric cancer in all cases and different age and gender subgroups were analyzed by multivariate logistic regression analysis. In the patient cohort of gastric cancer, receiver-operating characteristic analyses were performed to calculate the best cutoff values of serum folate, and the associations between serum folate levels and clinicopathological features were further analyzed by multivariate regression analysis. Survival analyses were conducted using the Cox proportional hazards model.

RESULTS: The mean serum folate level was significantly lower in gastric cancer patients than that in controls (3.71 ± 0.30 ng/mL *vs* 8.00 ± 0.54 ng/mL, $P < 0.01$), and folate levels were consistently lower in gastric cancer patients regardless of age and gender (all $P < 0.01$). Using the median serum folate value as the cutoff value, low serum folate was significantly associated with gastric cancer risk in the whole population (OR = 19.77, 95%CI: 10.54-37.06, $P < 0.001$) and all strata (age < 60 years OR = 17.39, 95%CI: 7.28-41.54, age ⩾ 60 years (OR = 21.67, 95%CI: 8.27-56.80), males (OR = 17.95, 95%CI: 7.93-40.62), and females (OR = 20.95, 95%CI: 7.66-57.31); all $P < 0.001$. In the patient cohort of gastric cancer, the respective cutoff values showed that low serum folate levels were significantly associated with serosal invasion (OR = 2.54, 95%CI: 1.23-5.23), lymphatic invasion (OR = 2.23, 95%CI: 1.17-4.26), and liver metastasis (OR =

6.67, 95%CI: 1.28-34.91) of gastric cancer (all $P < 0.05$). Serum folate level below 1.90 ng/mL was associated with poor patient survival (HR = 1.84, 95%CI: 1.04-3.27, $P < 0.05$) in univariate analysis.

CONCLUSION: Lower serum folate levels were significantly associated with gastric cancer development and invasive phenotypes. The role of folate depletion in gastric cancer invasion warrants further study.

Key words: Folic acid; Folate; Plasma; Metastasis; Invasion

Core tip: Low folate status is involved in the development of gastric cancer, but the role of folate in invasiveness of gastric cancer remains unclear. In addition, although folate levels in blood may reflect the degree of folate depletion, an association between blood folate status and gastric cancer has not been established. In this case-control study, we found lower serum folate was significantly associated with gastric cancer development. Besides, in the patient cohort of gastric cancer, lower serum folate was significantly associated with invasive phenotypes such as serosal invasion, lymphatic invasion and liver metastasis. These findings warrant further study.

Lee TY, Chiang EP, Shih YT, Lane HY, Lin JT, Wu CY. Lower serum folate is associated with development and invasiveness of gastric cancer. *World J Gastroenterol* 2014; 20(32): 11313-11320 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11313.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11313

INTRODUCTION

Gastric cancer remains the fourth most common cancer and the second leading cause of cancer mortality worldwide, and the median survival in patients with advanced (stages Ⅲ-Ⅳ) gastric cancer is generally less than one year[1-3]. Due to poor treatment response in advanced gastric cancer, detection of early-stage gastric cancer can effectively improve outcomes[4]. However, early diagnosis remains a challenge in most countries due to invasive characteristic of gastroscopy and a lack of practical screening biomarkers, and most patients already suffer from advanced tumor when gastric cancers are newly diagnosed[5]. In addition, the optimal degree of lymph node dissection for gastric cancer is still matter of debate, and inaccurate pre-operative image studies of lymph node metastasis frequently result in over- or incomplete resection[6]. New biomarkers to screen for and predict the invasiveness of gastric cancer may provide novel ways to resolve the dilemma and are therefore urgently needed[7,8].

Folate is involved in biological methylation reactions and nucleotide biosynthesis, and depletion of folate can result in global DNA hypomethylation, DNA damage, impaired DNA repair and altered proto-oncogene/tumor suppressor gene expressions[9,10]. Folate supplementation was recently reported to significantly increase global DNA methylation and reduce mucosal inflammation and dysplasia in a *Helicobacter pylori*-infected mouse model, and gastric cancer may be prevented by high folate intake[11]. Although the results of studies on dietary folate intake and risk of gastric cancer were inconsistent[12-14], gene 677CT polymorphism of methylenetetrahydrofolate reductase (MTHFR), a key enzyme in the metabolism of folate, was associated with increased risk of gastric cancer for individuals with low folate status[15,16]. Moreover, folate concentrations were reported to be significantly lower in gastric cancer tissues than in gastritis tissues, and hypomethylation and overexpression of c-myc were correlated with lower folate concentrations in tissues[17]. Increasing evidence suggests low folate status is involved in the development of gastric cancer, but the role of folate in gastric cancer invasiveness remains unclear.

Although folate levels in blood may reflect the degree of folate depletion, an association between blood folate status and gastric cancer has not been established. In a study of advanced gastric cancer, plasma folic acid concentration was lower in patients with total genomic DNA hypomethylation than that in patients with normal methylation[18]. Tan *et al*[19] observed that the mean serum folate concentration of gastric cancer cases was significantly lower than that of controls. However, Vollset *et al*[20] reported a null association between plasma folate levels and gastric cancer risk, but a mean duration of 3.3 years between blood donation and cancer diagnosis could have biased the observed results. The serial changes of serum folate were not reported when patients developed gastric cancer, and further studies to verify lower blood folate in gastric cancer patients are needed.

To our knowledge, the associations between serum folate levels, invasive phenotypes and patient survival of gastric cancer have not been evaluated. Therefore, we conducted a study to determine whether serum folate level could be clinically associated with development, invasiveness and patient survival of gastric cancer.

MATERIALS AND METHODS

Study subjects

In this case-control study, we used blood samples collected from individuals participating in two national projects conducted from January 1998 to April 2006 for the investigation of risk factors of gastric cancer in Taiwan[21,22]. Patients with newly diagnosed gastric cancer undergoing gastrectomy were enrolled, and patients receiving chemotherapy prior to surgery, with other concurrent malignancy, or of the aboriginal and alien populations were excluded. Control blood samples were obtained from individuals who visited health examination clinics with minimal gastritis or normal appearance of the gastric

Table 1 Demographic data and folate concentrations of gastric cancer patients and controls

	Case (n = 155)	Control (n = 149)	P value[1]
Age (yr)	62.02 ± 1.14	57.21 ± 0.86	< 0.01
Gender n (%)			
Male	88 (56.8)	90 (60.4)	NS
Female	67 (43.2)	59 (39.6)	
H. pylori infection n (%)			
No	83 (53.5)	78 (52.3)	NS
Yes	72 (46.5)	71 (47.7)	
Folate (ng/mL)	3.71 ± 0.30	8.00 ± 0.54	< 0.01
Male	3.12 ± 0.34	7.34 ± 0.74	< 0.01
Female	4.49 ± 0.54	9.01 ± 0.76	< 0.01
Age ⩾ 60	3.70 ± 0.42	9.87 ± 1.17	< 0.01
Age < 60	3.72 ± 0.43	6.78 ± 0.43	< 0.01

[1]All P values ⩾ 0.05 were considered to be statistically non-significant (NS).

mucosa on gastroscopic examination. Informed consents were obtained from all subjects and/or guardians on a voluntary basis, and all patient-derived specimens were collected under protocols approved by the Institutional Review Boards (IRBs) of the parent institutions (IRB No. C07199). All the study subjects who fulfilled the inclusion and exclusion criteria and signed the informed consents were recruited.

In total, we studied 155 patients with gastric cancer, for whom complete clinical data, including tumor stage, degree of tumor invasion and presence of metastasis, and a serum sample were available. All recruited patients had been followed up for at least 5 years. The controls were matched by age (within 5 years) and date of blood collection (± 3 mo), and 149 cases were recruited. In addition, status of *Helicobacter pylori* (*H. pylori*) infection was determined by Giemsa staining of gastric tissue biopsy or by serum *H. pylori* antibody test (INOVA Diagnostics, San Diego, CA).

Measurement of serum folate levels

Blood samples were obtained after overnight fasting, and serum folate concentrations were measured by microbiological assay[23]. Cryopreserved *Lactobacillus casei* (NCIB 10463) culture were thawed into reconstituted assay medium in a water bath at 37 ℃, and then added to bulk assay medium at a concentration of 200 μL/100 mL. Sodium ascorbate (0.5%) solution was used to dilute serum samples 1:20. Standard solution of folate (500 pg/mL) was made by diluting stock standards in sodium ascorbate. Assay microorganism was added, and the plates were sealed and incubated at 37 ℃ in the dark for 42 h after adding disinfectant. Then they were read at 570 nm and serum folate concentrations were calculated.

Statistical analysis

The discrete variables are presented as number and percentage (%); continuous variables are presented as mean and standard error. The demographic data of patients and controls were compared using the χ^2 test and Student's t-test. To avoid possible confounding effects of age and gender, multivariate logistic regression analyses were conducted to evaluate the associations between serum folate levels, gastric cancer risk, and clinicopathological features. Using the median value of serum folate computed among the overall population of cases and control as the cutoff value, the associations between serum folate and gastric cancer in all cases and different age and gender subgroups were analyzed. In the patient cohort of gastric cancer, we considered that the cutoff values in various clinicopathological features should be different, so nonparametric receiver-operating characteristics (ROC) analyses were plotted for determining the best cutoff values of serum folate in various clinicopathological features. Using the respective cutoff values, the associations between serum folate levels and clinicopathological features were analyzed. Furthermore, to determine whether serum folate concentration was associated with patients' outcomes, survival analyses were conducted using the Cox proportional hazards model. Data were analyzed using SPSS, version 11.0 (SPSS Inc., Chicago, Illinois, United States). Nonparametric ROC analyses were performed via the STATA program, version 8.0 (Stata Corporation, College Station, Texas, United States).

RESULTS

Association between serum folate levels and gastric cancer

Demographic characteristics are summarized in Table 1. Control subjects were on average about 5 years younger than gastric cancer cases. There were no differences in gender distribution or *H. pylori* infection status between the two groups. The mean serum folate level was significantly lower in gastric cancer patients compared to that in controls (3.71 ± 0.30 ng/mL *vs* 8.00 ± 0.54 ng/mL, P < 0.01). Since gastric cancer patients were slightly older than controls, we further analyzed folate concentrations based on age and gender, and found folate levels were consistently lower in gastric cancer patients regardless of age and gender (all P < 0.01).

Using the median value of serum folate (4.38 ng/mL) computed among the overall population of cases and control as the cutoff value, the adjusted odds ratio (OR) for detecting the occurrence of gastric cancer was 19.77 (95%CI: 10.54-37.06) (Table 2). In addition, serum folate level lower than 4.38 ng/mL was found to be consistently associated with higher gastric cancer risk in all strata (age < 60 years, age ⩾ 60 years, males, and females; all P < 0.001). No significant interaction of other variables was observed.

Association between serum folate levels and invasiveness of gastric cancer

To further assess the value of using serum folate levels for detecting invasiveness among gastric cancer patients, we calculated the best cutoff values of various invasive phenotypes by ROC analyses. Using the cutoff values for multivariate regression analyses, the adjusted ORs for detecting the invasiveness of gastric cancer were obtained,

Table 2 Association between serum folate level and occurrence of gastric cancer *n* (%)

	Folate		Odds ratio[1]	
	> 4.38 ng/mL	⩽ 4.38 ng/mL	95%CI	*P* value
All cases				
Control (*n* = 149)	119 (79.9)	30 (20.1)	1	
Gastric cancer (*n* = 155)	33 (21.3)	122 (78.7)	19.77 (10.54-37.06)	< 0.001
Age < 60 (yr)				
Control (*n* = 90)	70 (77.8)	20 (22.2)	1	
Gastric cancer (*n* = 59)	10 (16.9)	49 (83.1)	17.39 (7.28-41.54)	< 0.001
Age ⩾ 60 (yr)				
Control (*n* = 59)	49 (83.1)	10 (16.9)	1	
Gastric cancer (*n* = 96)	23 (24.0)	73 (76.0)	21.67 (8.27-56.80)	< 0.001
Male				
Control (*n* = 90)	68 (75.6)	22 (24.4)	1	
Gastric cancer (*n* = 88)	14 (15.9)	74 (84.1)	17.95 (7.93-40.62)	< 0.001
Female				
Control (*n* = 59)	51 (86.4)	8 (13.6)	1	
Gastric cancer (*n* = 67)	19 (28.4)	48 (71.6)	20.95 (7.66-57.31)	< 0.001

[1]Age and gender were adjusted by multivariate regression analysis.

Table 3 Associations between serum folate levels and clinicopathological features of gastric cancer *n* (%)

	Folate		Odds ratio[1]	
	> 2.61 ng/mL	⩽ 2.61 ng/mL	95%CI	*P* value[2]
Stage				
Early (*n* = 28)	14 (50.0)	14 (50.0)	1	
Advanced (*n* = 127)	54 (42.5)	73 (57.5)	1.38 (0.60-3.14)	NS
Serosal invasion				
Absent (*n* = 43)	26 (60.5)	17 (39.5)	1	
Present (*n* = 112)	42 (37.5)	70 (62.5)	2.54 (1.23-5.23)	< 0.05
Lymph node metastasis				
Absent (*n* = 51)	26 (51.0)	25 (49.0)	1	
Present (*n* = 104)	42 (40.4)	62 (59.6)	1.54 (0.78-3.02)	NS
Venous invasion				
Absent (*n* = 90)	42 (46.7)	48 (53.3)	1	
Present (*n* = 65)	26 (40.0)	39 (60.0)	1.32 (0.69-2.52)	NS
Lymphatic invasion				
Absent (*n* = 74)	40 (54.1)	34 (45.9)	1	
Present (*n* = 81)	28 (34.6)	53 (65.4)	2.23 (1.17-4.26)	< 0.05
Liver metastasis	(> 1.9 ng/mL)	(⩽ 1.90 ng/mL)		
Absent (*n* = 146)	104 (71.2)	42 (28.8)	1	
Present (*n* = 8)	2 (25.0)	6 (75.0)	6.67 (1.28-34.91)	<0.05

[1]Age and gender were adjusted by multivariate regression analysis; [2]All *P* values ⩾ 0.05 were considered to be statistically non-significant (NS).

as shown in Table 3. Serum folate ⩽ 2.61 ng/mL was significantly associated with serosal invasion (OR = 2.54, 95%CI: 1.23-5.23) and lymphatic invasion (OR = 2.23, 95%CI: 1.17-4.26). Serum folate ⩽ 1.90 ng/mL was significantly correlated with liver metastasis (OR = 6.67, 95%CI: 1.28-34.91) (all $P < 0.05$).

Association between serum folate levels and patient survival

The survival curves for patients with different serum folate levels are compared in Figure 1. A folate level lower than 1.90 ng/mL was associated with poor survival ($P = 0.03$). Cox proportional hazard model analyses showed that advanced stage ($P < 0.001$), serosal invasion ($P < 0.001$), lymph node metastasis ($P < 0.001$), lymphatic invasion ($P < 0.001$), venous invasion ($P < 0.001$), liver metastasis ($P < 0.001$), and serum folate level lower than 1.90 ng/mL ($P = 0.036$) were associated with poor overall survival (Table 4). Multivariate analysis showed that only serosal invasion ($P < 0.001$) and liver metastasis ($P < 0.001$) were independent risk factors for poor overall survival.

DISCUSSION

Previous studies have shown that low folate status is involved in the development of gastric cancer[15-17], but the role of folate in the invasiveness of gastric cancer is still unclear. To our knowledge, this is the first study to investigate whether serum folate could be clinically associated with invasiveness of gastric cancer. We found low serum folate levels were significantly associated with various invasive phenotypes such as serosal invasion, lymphatic invasion and liver metastasis. The findings of our study suggest that folate depletion may play a role in the development of gastric cancer, but the effect of folate depletion in gastric cancer invasion warrants further investigation.

Although folate supplementation has been shown to be effective in preventing global loss of methylation in a mouse model of gastric cancer development[11], further study will be required to determine whether folate depletion and subsequent perturbed hypomethylation directly contribute to tumor invasion and metastasis. In a recent study, Wang *et al*[24] found that folate deficiency could enhance invasiveness of colon cancer cells by activation of Shh signaling through promoter hypomethylation and cross actions with the NF-κB pathway. However, the effect of folate deficiency on methyl group metabolism and methylation is highly complex, and is influenced by cell type, stage of transformation, and genetic variations at specific sites in the genome[25-28]. For example, in animal studies of colorectal cancer, although folate showed chemoprotective effects against carcinogenesis, it also appeared to promote progression of cancer once neoplastic foci had been established[29-31]. The mechanisms of folate deficiency that contribute to the invasiveness of gastric cancer have yet to be fully elucidated.

Few studies have investigated the association between

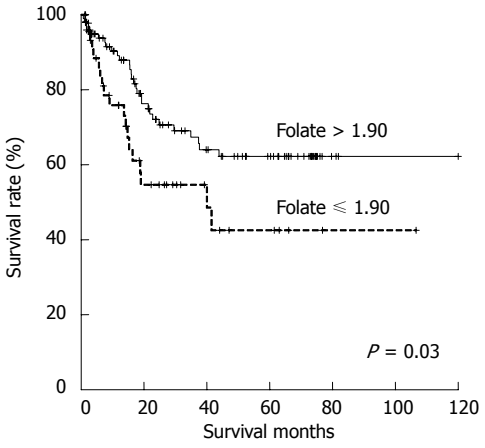

Figure 1 Survival curves for patients with different serum folate levels. The survival rate for those with lower folate levels was significantly lower compared with those with higher folate levels ($P = 0.03$).

Table 4 Univariate analysis of mortality predictors in gastric cancer patients

	Hazard ratio	95%CI	*P* value[1]
Age			
> 60 *vs* ⩽ 60	1.27	0.97-1.67	NS
Gender			
Male *vs* female	1.29	0.98-1.69	NS
Stage			
Advanced *vs* early	7.78	3.96-15.26	< 0.001
Depth of invasion			
Serosal *vs* non-serosal invasion	3.96	2.67-5.89	< 0.001
Lymph node metastasis			
Yes *vs* no	2.47	1.78-3.43	< 0.001
Lymphatic invasion			
Yes *vs* no	2.17	1.53-3.07	< 0.001
Venous invasion			
Yes *vs* no	2.45	1.73-3.46	< 0.001
Liver metastasis			
Yes *vs* no	6.01	4.04-8.93	< 0.001
Folate			
⩽ 1.90 *vs* > 1.90 ng/mL	1.84	1.04-3.27	0.036

[1]All *P* values ⩾ 0.05 were considered to be statistically non-significant (NS).

blood folate status and gastric cancer development, and the usefulness of blood folate as a biomarker is still a subject of debate. A Chinese case-control study showed that gastric cancer patients had a significantly lower serum folate level than that of controls, but a European study found there was no significant difference in plasma folate level between pre-gastric cancer patients and the controls[19,20]. However, in that European study, the blood samples were collected 3.3 years (mean, with a range of 0.4-7.13 years) before diagnosis of gastric cancer, and this discrepancy could be biased by the time interval between blood donations and cancer diagnosis. In addition, other risk factors may be also involved in the discrepancy between Chinese and European populations. For example, in a recent meta-analysis[32], the gene polymorphism in thymidylate synthase, an important enzyme involved in folate metabolism, was associated with an increased risk of gastroesophageal cancer among Asians, but not among Caucasians. However, the interaction between other risk factor and blood folate in different populations warrants further evaluation. In the present study of Chinese population, we confirmed that the serum folate level was significantly lower in patients than that in controls, and low serum folate was significantly associated with increased gastric cancer risk regardless of age and gender. According to the observations of previous studies and our results, decreased folate levels can be found after the development of gastric cancer. Further studies to confirm the causal relationship between folate depletion and gastric cancer evolution will be crucial.

The association between folate and outcomes of gastric cancer has rarely been investigated, although MTHFR 677TT carriers with low folate and vitamin B12 intakes were reported to have the lowest survival rate in a cohort study of gastric cancer[33]. The present investigation is the first to report that a low serum folate level was associated with poor patient survival in univariate analysis, but the association became insignificant in multivariate analysis. Although low serum folate level was not associated with prognosis in this study, outcome analysis could be biased by different strategies and clinical conditions in cancer treatment. Large-scale well-controlled prospective studies will be needed to determine the value of serum folate level as a prognostic biomarker.

Decreased serum folate levels may be caused by poor intake of folate-rich foods, impaired folate absorption, impaired folate metabolism or rapid folate consumption. Interestingly, the role of dietary folate intake in the development of gastric cancer has been reported in several studies, with controversial results[12-14]. Larsson *et al*[12] conducted a meta-analysis and found significant heterogeneity, especially among different geographic regions. The estimated relative risks for subjects with the highest dietary folate intake relative to the lowest dietary folate intake were 0.68 (95%CI: 0.58-0.80) for studies conducted in the United States, 1.15 (95%CI: 0.91-1.45) for European studies, and 0.89 (95%CI: 0.40-1.96) for studies conducted elsewhere. The disparity in these results may be due to complex host-environment interactions. Moreover, a variety of conditions, including defects in the uptake system, gastrointestinal diseases, drug interaction, and hypochlorhydria may also affect the normal intestinal folate absorption process[34,35]. Gastric cancer arises by a multi-stage process, and severity of atrophic gastritis is correlated to the risk of progression to gastric cancer[36]. Although low serum folate in patients with gastric cancer may be caused by disturbed folate absorption due to severe underlying atrophic gastritis, the possible mechanisms involved in folate depletion may be complicated and should be further clarified.

The association between *H. pylori* infection and folate depletion has been evaluated in previous studies. In a recent meta-analysis[35], no significant association between *H. pylori* infection and folate levels was observed, and eradication of *H. pylori* infection had no effect on serum folate levels. In this case-control study, the impact of *H.*

pylori infection on serum folate levels was also not significant. However, the proportions of patients with *H. pylori* infection were similar in the gastric cancer group and the matched control group, and the prevalence of *H. pylori* infection in the gastric cancer group may be underestimated due to *H. pylori* eradication therapy and lower sensitivity of *H. pylori* test in severe atrophic gastritis[37]. Further study may help to clarify the effect of *H. pylori* infection on folate levels among gastric cancer patients.

There are several limitations to this study. First, although selection bias could not be completely excluded, we enrolled study subjects in a multi-center setting. This should help minimize the selection bias. Second, despite the fact that a causal relationship between folate depletion and gastric cancer evolution could not be determined in this clinical study; we provided important clues for further study. Third, although the controls were matched by age (within 5 years) in the present study, gastric cancer patients were significantly older than the controls. Using multivariate regression and subgroup analyses, we didn't observe significant correlation with age. Fourth, some important information could not be fully obtained in this retrospective study; for example, body mass index (BMI). Even though the mean BMI values of study subjects with available data were not significantly different between the gastric cancer group and the control group, we did not present the findings because BMI data for some subjects were missing. A prospective study could provide a more complete description of study population. Finally, although we did not analyze other environmental factors, such as cigarette smoking or alcohol consumption in this retrospective study, associations between lower blood folate levels and these environmental factors in gastric cancer patients were not reported in previous studies[19]. Further prospective control studies are needed to clarify the effects of other risk factors.

The present study demonstrated several important findings: first, lower serum folate was observed in gastric cancer patients. Second, we found strong relationships between lower serum folate and various invasive phenotypes, including serosal invasion, lymphatic invasion and liver metastasis in gastric cancer. Third, serum folate level was demonstrated to be a potentially useful biomarker of gastric cancer progression in terms of occurrence, serosal invasion, lymphatic invasion and liver metastasis, but further studies are needed to confirm that serum folate is an important biomarker for gastric cancer. In conclusion, our study findings suggest that folate depletion may play a role in the development and invasiveness of gastric cancer, but the effect of folate depletion in gastric cancer invasion warrants further study.

COMMENTS

Background

Treatment response in advanced gastric cancer is usually poor, but detection of early-stage gastric cancer can effectively improve outcomes. Early diagnosis remains a challenge, and most patients already suffer from advanced tumor when gastric cancers are newly diagnosed. In addition, inaccurate pre-operative image studies of lymph node metastasis frequently result in over- or incomplete resection. New biomarkers to screen for and predict the invasiveness of gastric cancer are urgently needed.

Research frontiers

Folate is involved in biological methylation reactions and nucleotide biosynthesis, and depletion of folate can result in carcinogenesis. Folate supplementation was recently reported to significantly increase global DNA methylation and reduce mucosal inflammation and dysplasia, and gastric cancer may be prevented by high folate intake. Increasing evidence suggests low folate status is involved in the development of gastric cancer, but the role of folate in gastric cancer invasiveness remains unclear.

Related publications

An association between blood folate status and gastric cancer has not been established. The mean serum folate concentration of gastric cancer cases was significantly lower than that of controls in a Chinese study, but a null association between plasma folate levels and gastric cancer risk was reported in a European study. The associations between serum folate levels, invasive phenotypes and patient survival of gastric cancer have not been evaluated.

Innovations and breakthroughs

In this case-control study, authors found lower serum folate was significantly associated with gastric cancer development. Besides, in the patient cohort of gastric cancer, lower serum folate was significantly associated with invasive phenotypes such as serosal invasion, lymphatic invasion and liver metastasis. However, serum folate level was not associated with patient survival.

Applications

Serum folate level was demonstrated to be a potentially useful biomarker of gastric cancer progression in terms of occurrence, serosal invasion, lymphatic invasion and liver metastasis, but further studies are needed to confirm that serum folate is an important biomarker for gastric cancer.

Terminology

Folate is a naturally occurring form of the vitamin B that can be found in food. Depletion of folate can result in global DNA hypomethylation, DNA damage, impaired DNA repair and altered proto-oncogene/tumor suppressor gene expressions.

Peer review

This is valuable research which addresses an important topic. In this case-control study, the serum folate level was significantly lower in gastric cancer patients than that in controls, and folate levels were consistently lower in gastric cancer patients regardless of age and gender. In the patient cohort of gastric cancer, low serum folate levels were significantly associated with invasive phenotypes of gastric cancer. Findings of this study suggest that folate depletion may play a role in the development and invasiveness of gastric cancer, but the effect of folate depletion in gastric cancer invasion warrants further investigation.

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World J Gastroenterol 2014 August 28; 20(32): 11321-11325
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

RETROSPECTIVE COHORT STUDY

Electrocardiograms changes in children with functional gastrointestinal disorders on low dose amitriptyline

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Received: December 20, 2013 Revised: March 28, 2014
Accepted: May 28, 2014
Published online: August 28, 2014

Abstract

AIM: To study the effects of low dose amitriptyline on cardiac conduction in children.

METHODS: Secondary analysis of data obtained from a double-blind, randomized placebo-controlled trial, evaluating low dose amitriptyline in children with a diagnosis of functional abdominal pain, functional dyspepsia, and irritable bowel syndrome according to the Rome II criteria. Children 8-17 years of age were recruited from the pediatric gastroenterology clinics of 6 tertiary care centers in the United States. The electrocardiograms (EKGs) done prior to initiation of amitrityline and 1 mo after initiation of amitriptyline were examined. The changes in cardiac conduction were evaluated in patients and controls.

RESULTS: Thirty children were included in the study. There were 12 patients, ages 9-17 years of both genders, in the amitriptyline treatment group and 18 patients, ages 9-17 years of both genders, in the placebo treatment group. None of the patients had any baseline EKG abnormality. Amitriptyline use was associated with an increase in heart rate (P = 0.024) and QTc interval (P = 0.0107) as compared to pre-EKGs. Children in the placebo group were also noted to present a statistically significant increase in QTc interval (P = 0.0498). None of the patients developed borderline QTc prolongation or long-QT syndrome after they were started on amitriptyline.

CONCLUSION: The study findings suggest that once patients with functional gastrointestinal disorders have been screened for prolonged QTc interval on baseline EKG, they probably do not need a second EKG for re-evaluation of cardiac conduction after starting low dose amitriptyline.

Key words: Amitriptyline; Electrocardiogram; Children; Abdominal pain related-functional gastrointestinal disorders

Core tip: Information on electrocardiogram changes in children who are on low dose amitriptyline for treatment of abdominal pain associated-functional gastrointestinal disorders (AP-FGIDs) is sparse. To better understand the effects of low dose amitriptyline on cardiac conduction in children, we reviewed the electrocardiogram findings before and after initiation of amitriptyline. We found that use of low dose amitriptyline in children with AP-FGIDs was not associated with clinically significant changes in cardiac conduction.

Chogle A, Saps M. Electrocardiograms changes in children with functional gastrointestinal disorders on low dose amitriptyline. *World J Gastroenterol* 2014; 20(32): 11321-11325 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11321.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11321

INTRODUCTION

Abdominal pain associated-functional gastrointestinal disorders (AP-FGIDs) are among the most common medical afflictions in childhood and adolescence[1,2]. The AP-FGIDs have been categorized by the Rome Ⅲ criteria into irritable bowel syndrome (IBS), functional abdominal pain (FAP), functional dyspepsia (FD) and abdominal migraine (AM). These disorders have been shown to significantly affect the children's quality of life[1,2]. Tricyclic antidepressants (TCAs) like amitriptyline have played an important role in the treatment of pediatric psychiatric disorders. However, the increased risk of adverse cardiac events, sudden deaths in children and a black box warning by the FDA on the risk of suicidality have led to a decline in their use[3,4]. TCAs have now been relegated to second line status for treatment of depression. Studies in adults have shown benefit in using TCAs for treatment of AP-FGIDs[5-11]. Amitriptyline has not been found to be better than placebo for treatment of AP-FGIDs in children and is not approved for the treatment of FGIDs in children or adolescents, but its off label use is prevalent[12-15]. The dose of amitriptyline commonly used in the treatment of FGIDs in children is much lower than that used for depression. A dose-response association of TCAs has been demonstrated with QTc prolongation[16]. TCA overdose has been shown to cause conduction delays and prolonged corrected QT (QTc) in children[17]. There is limited data in the literature regarding the cardiac toxicity with therapeutic antidepressant doses of amitriptyline in children. Information on electrocardiogram (EKG) changes in children who are on low dose amitriptyline for treatment of FGIDs is sparse. To better understand the effects of low dose amitriptyline on cardiac conduction in children, we reviewed the EKG findings before and after initiation of amitriptyline in a multicenter study conducted by our group assessing the efficacy of amitriptyline in children with AP-FGIDs[14].

MATERIALS AND METHODS

This study was a secondary analysis of data obtained from a double-blind, randomized placebo-controlled trial, evaluating amitriptyline in children with a diagnosis of functional abdominal pain, functional dyspepsia, and IBS according to the Rome II criteria[14]. In the original study children 8-17 years of age were recruited from the pediatric gastroenterology clinics of 6 tertiary care centers geographically dispersed in the United States: Children's Hospital of Pittsburgh (Pittsburgh, PA), Goryeb Children's Hospital at Atlantic Health System (Morristown, NJ), Kansas University Medical Center (Kansas City, KS), Children's Hospital of Boston (Boston, MA), Children's Hospital of Wisconsin (Milwaukee, WI), and Children's Memorial Hospital (Chicago, IL). In the current study only those patients in both groups (amitriptyline treatment and placebo groups) who had a standard 12-lead EKG before starting the study medication (pre-EKGs) and a second EKG 1 mo after initiation of the medication (post-EKGs) were included. Only the centers located at Chicago, Pittsburgh and Boston required EKGs to be done prior to and after starting amitriptyline. Amitriptyline was dosed based on their weight: (1) < 35 kg-10 mg capsule by mouth daily; and (2) ⩾ 35 kg-20 mg capsule by mouth daily. All EKGs were read by pediatric cardiologists at the respective study locations. We examined the effect of amitriptyline and placebo on heart rate (HR), PR, QRS, and QTc interval with the 2 tailed *t*-test using GraphPad statistical software.

A QTc interval at or above 480 ms in females or 470 ms in males was considered diagnostic of long-QT syndrome (LQTS)[14]. The diagnosis of borderline QT prolongation was given when a patient had a QTc value between 440 and 470 ms.

RESULTS

Thirty children were included in the study. There were 12 patients (10 females), ages 9-17 years (10 patients were 10 years or older), in the amitriptyline treatment group and 18 patients (15 females), ages 9-17 years (16 patients were 10 years or older), in the placebo treatment group. Using the D'Agostino-Pearson test for normality, the data was found to have normal distribution. None of the patients had any baseline EKG abnormality. Amitriptyline use was associated with an increase in heart rate (P = 0.024) and QTc interval (P = 0.0107) as compared to pre-EKGs (Table 1). Children in the placebo group were also noted to present a statistically significant increase in QTc interval (P = 0.0498) (Table 1). None of the patients developed borderline QTc prolongation or LQTS after they were started on amitriptyline.

DISCUSSION

Amitriptyline at low doses is thought to work primarily by inducing pain tolerance through peripheral or central anti-nociceptive properties as well through its anticholinergic effects, and secondarily through its anxiolytic effects[8,14,18].

A meta-analysis of adult studies showed that amitriptyline is beneficial in treatment of FGIDs in adults[5,7,8,19]. There have been 2 randomized controlled pediatric trials that have examined the efficacy and safety profile of low dose amitriptyline in treatment of FGIDs[13,14]. Both studies found no statistically significant differences between amitriptyline and placebo for most efficacy outcomes including improvement of abdominal pain (Bahar study found improvement exclusively in RLQ pain and a beneficial effect in quality of life). A review by the Cochrane's Group concluded that there was no evidence to support the use of amitriptyline for the treatment of abdominal pain-related FGIDs in children and adolescents[12]. Despite the lack of evidence of its efficacy, clinicians commonly prescribe amitriptyline to children with AP-FGIDs. Typically a 0.5-1 mg/kg per day dose of amitrip-

Table 1 Effect of amitriptyline and placebo on cardiac conduction in children

Parameters	HR (beats/min)		PR (ms)		QRS (ms)		QTc (ms)	
	Pre EKG	Post EKG	Pre EKG	Post EKG	Pre EKG	Post EKG	Pre EKG	Post EKG
Drug								
mean ± SD	75.92 ± 9.44	85.2 ± 17.4	135 ± 12.97	134.5 ± 11.25	83.67 ± 6.81	85.33 ± 7.15	406.91 ± 12.6	418 ± 13.8
2 tailed *t* test *P* value	0.024[a]		0.8429		0.2098		0.0107[a]	
Correlation coefficient - *r*	0.7367		0.7608		0.8084		0.4923	
Placebo								
mean ± SD	69.22 ± 12.26	74.05 ± 10.06	132.6 ± 16.35	137.5 ± 21.53	86.7 ± 8.95	83.8 ± 8.98	415.5 ± 16.82	422.6 ± 18.53
n	18	18	18	18	18	18	18	18
2 tailed *t* test *P* value	0.0783		0.1394		0.0937		0.0498[a]	
Correlation coefficient - *r*	0.5343		0.7841		0.7037		0.6773	

[a]$P < 0.05$, placebo group *vs* control group.

tyline to a maximum of 50 mg daily is used as opposed to 1-3 mg/kg per day used for depression in children[14].

Amitriptyline activates cardiac ryanodine channels causing efflux of calcium from the sarcoplasmic reticulum[20]. As a result, amitriptyline is pro-arrhythmogenic and increases the risk of sudden cardiac death in patients with underlying heart disease and at doses above 100 mg daily[3]. Amitriptyline has been classified as a "conditional risk" drug for development of torsade de pointes[21]. Drugs with "conditional risk" have significant evidence of prolonging QT and causing torsade de pointes but only under certain conditions, such as excessive dose or drug interaction[22]. Some studies have documented changes in EKG tracings in children on the higher doses of amitriptyline used for depression[23,24]. These have included findings ranging of no changes in any of the tracings to increases in the heart rate, PR, QRS and QTc intervals. In a recent risk prevention study, the incidence of prolonged QTc interval in a subpopulation of children with IBS before the initiation of amitriptyline was found to be 0.4%, which is similar to the incidence of prolonged QTc in adult and adolescent athletes[25,26]. This study concluded that a screening EKG should always be performed on children with FGIDs, before initiating amitriptyline therapy[25].

We found practice variation in conducting EKGs after starting patients on amitriptyline, with only 3 of the 6 tertiary centers doing the pre and post amitriptyline initiation EKGs. This practice variation likely exists due to paucity of data on the effect of low dose amitriptyline on cardiac conductance. Our study shows that post amitriptyline EKGs might not be necessary. The cost of performing an EKG as per CMS reimbursement schedule ranges from $39-47. For every additional EKG there are additional intangible costs such as lost wages for parents, childcare costs for siblings, cost of transportation, etc. Performing unnecessary EKGs after initiating amitriptyline can add to the existing lofty healthcare expenditure in managing children with FGIDs, especially considering the high prevalence of chronic abdominal pain in school age children[1,27].

The chronic use of TCAs in adult patients with chronic pain was not shown to result in clinically significant changes in cardiac conduction[28]. In our study amitriptyline use was associated with an increase in the heart rate and the QTc interval but none of these changes were significant enough to warrant discontinuation of amitriptyline. These findings suggest that once patients with FGIDs have been screened for prolonged QTc interval on baseline EKG, they probably do not need a second EKG for reevaluation of cardiac conduction after starting low dose amitriptyline. Limitations of our study include the small sample size. Studies with larger sample size and longer duration are needed to confirm our findings and possibly influence future monitoring recommendations post initiation of TCA for FGID treatment. The findings might not be generalizable to other TCAs. The frequent use of TCAs other than amitriptyline for the treatment of FGIDs in children stress the importance of conducting similar studies with other TCAs[15].

Our preliminary study suggests that amitriptyline used in low doses can be considered a relatively safe drug in the arsenal of pediatric gastroenterologists.

Our retrospective placebo controlled study suggests that the use of low dose amitriptyline in children with AP-FGIDs is probably not associated with clinically significant changes in cardiac conduction. A larger prospective study should be designed to confirm these findings.

COMMENTS

Background

Abdominal pain associated-functional gastrointestinal disorders (AP-FGIDs) commonly occur in childhood and adolescence. Low dose Amitriptyline is commonly used for off label treatment of AP-FGIDs in children although it has not been found to be better than placebo nor is it not approved for the treatment of FGIDs in children or adolescents. A dose-response association of tricyclic antidepressants has been demonstrated with QTc prolongation. There is limited data in the literature regarding the cardiac toxicity of low dose Amitriptyline in children.

Research frontiers

Information on electrocardiogram (EKG) changes in children who are on low dose Amitriptyline for treatment of FGIDs is sparse. Authors present data for the first time, which examines the effect of low dose Amitriptyline on cardiac conduction.

Innovations and breakthroughs

The study shows that the use of low dose amitriptyline in children with AP-FGIDs is not associated with clinically significant changes in cardiac conduction.

Applications

Performing unnecessary EKGs after initiating amitriptyline can add to the exist-

ing lofty healthcare expenditure in managing children with FGIDs, especially considering the high prevalence of chronic abdominal pain in school age children. There is a lot of practice variation amongst physicians in ordering a post Amitriptyline initiation EKG. Their data has the potential to decrease healthcare expenditure and to reduce practice variation.

Terminology

AP-FGIDs are among the most common medical afflictions in childhood and adolescence The AP-FGIDs have been categorized by the Rome Ⅲ criteria into irritable bowel syndrome (IBS), functional abdominal pain, functional dyspepsia and abdominal migraine.

Peer review

The study was a secondary analysis of data obtained from a double-blind, randomized placebo-controlled trial, evaluating Amitriptyline in children with a diagnosis of functional abdominal pain, functional dyspepsia, and IBS according to the Rome II criteria. They aimed to better understand the effects of low dose Amitriptyline on cardiac conduction in children. They reviewed the EKG findings before and after initiation of Amitriptyline in a large multicenter study conducted by their group assessing the efficacy of Amitriptyline in children with AP-FGIDs. They found that none of the patients had any baseline EKG abnormality. None of the patients developed borderline QTc prolongation or LQTS after they were started on Amitriptyline. They conclude that the study findings suggest that once patients with FGIDs have been screened for prolonged QTc interval on baseline EKG, they probably do not need a second EKG for reevaluation of cardiac conduction after starting low dose amitriptyline.

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P- Reviewer: Bashashati M, Bhalla C, Magazzu G
S- Editor: Ma YJ **L- Editor**: A **E- Editor**: Zhang DN

World Journal of Gastroenterology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i32.11326

World J Gastroenterol 2014 August 28; 20(32): 11326-11332
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

RETROSPECTIVE STUDY

Hospitalization for variceal hemorrhage in an era with more prevalent cirrhosis

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Received: January 17, 2014 Revised: March 11, 2014
Accepted: May 23, 2014
Published online: August 28, 2014

Abstract

AIM: To examine hospitalization rates for variceal hemorrhage and relation to cause of cirrhosis during an era of increased cirrhosis prevalence.

METHODS: We performed a retrospective review of patients with cirrhosis and gastroesophageal variceal hemorrhage who were admitted to a tertiary care referral center from 1998 to 2009. Subjects were classified according to the etiology of their liver disease: alcoholic cirrhosis and non-alcoholic cirrhosis. Rates of hospitalization for variceal bleeding were determined. Data were also collected on total hospital admissions per year and cirrhosis-related admissions per year over the same time period. These data were then compared and analyzed for trends in admission rates.

RESULTS: Hospitalizations for cirrhosis significantly increased from 611 per 100000 admissions in 1998-2001 to 1232 per 100000 admissions in 2006-9 (P value for trend < 0.0001). This increase was seen in admissions for both alcoholic and non-alcoholic cirrhosis (P values for trend < 0.001 and < 0.0001 respectively). During the same time period, there were 243 admissions for gastroesophageal variceal bleeding (68% male, mean age 54.3 years, 62% alcoholic cirrhosis). Hospitalizations for gastroesophageal variceal bleeding significantly decreased from 96.6 per 100000 admissions for the time period 1998-2001 to 70.6 per 100000 admissions for the time period 2006-2009 (P value for trend = 0.01). There were significant reductions in variceal hemorrhage from non-alcoholic cirrhosis (41.6 per 100000 admissions in 1998-2001 to 19.7 per 100000 admissions in 2006-2009, P value for trend = 0.007).

CONCLUSION: Hospitalizations for variceal hemorrhage have decreased, most notably in patients with non-alcoholic cirrhosis, and this may reflect broader use of strategies to prevent bleeding.

Key words: Varices; Gastrointestinal bleeding; Cirrhosis; Hospitalization; Portal hypertension

Core tip: Strategies to prevent gastroesophageal variceal bleeding, a morbid complication of cirrhosis, have been largely unchanged for 15 years. With the rising burden of cirrhosis over this time, it might be predicted that there would be a parallel increase in hospitalization rates for this complication. The findings from this study show that hospitalization rates for variceal bleeding are in fact decreasing, specifically in non-alcoholic cirrhosis. This raises the possibility that reductions in hospital admissions for variceal bleeding are attributable to more widespread use of prophylactic measures, and that expansion of these measures in patients with alcoholic cirrhosis could further reduce hospitalizations.

Lim N, Desarno MJ, Lidofsky SD, Ganguly E. Hospitalization for variceal hemorrhage in an era with more prevalent cirrhosis. *World J Gastroenterol* 2014; 20(32): 11326-11332 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11326.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11326

INTRODUCTION

Chronic liver disease is currently the twelfth leading cause of death in the United States; most liver disease-related deaths are the result of complications of cirrhosis. In particular, in 2007, 14406 and 14759 deaths were attributable to alcoholic and non-alcoholic cirrhosis, respectively. This represented an increase of 3.4% since 2006[1].

Development of gastroesophageal varices is a serious complication of cirrhosis. At the time of diagnosis of cirrhosis, esophageal varices are present in almost half of all patients and develop at a rate of approximately 7% per year[2]. The 1-year rate of a first variceal hemorrhage is approximately 12%. The short term mortality rate associated with variceal hemorrhage is over 15% and can be as high as 30% in patients with decompensated (Child's Class C) cirrhosis[2]. Thus, prevention of variceal bleeding is a critical goal in the management of cirrhosis. The use of nonselective beta-adrenergic receptor blockade and endoscopic band ligation for prophylaxis of variceal hemorrhage emerged in the 1980s and mid-1990s, respectively[3-7]. These strategies have been associated with a decline in mortality related to esophageal hemorrhage[8].

During this same era, however, cirrhosis and its complications have become significantly more prevalent, with increases from 18% to over 50%[9,10]. In the face of this rise in the burden of cirrhosis, it is therefore unknown whether the use of currently available prophylactic strategies would offset a parallel expected increase in the rate of variceal hemorrhage. Insights into this issue come from an analysis of a large nationwide hospital discharge database[11]. In this study, the hospitalization rate for bleeding varices increased from 1988 to 1996 and then fell between 1996 and 2002. Although these findings suggested that the changes reflected advances in prophylactic strategies, the data were obtained over a time period when such strategies were evolving.

Strategies to prevent variceal bleeding were codified in the form of practice guidelines in 1997[12] and no new strategies have emerged since. Thus, if an ongoing decrease in the incidence of variceal bleeding was observed in a more recent era, the expectation is that this would reflect increased utilization of effective prophylactic strategies. To test this, we assessed the rates of hospital admissions for variceal hemorrhage during the time period when prophylactic strategies for prevention of variceal hemorrhage had been well established (1998 to 2009). In addition, to our knowledge, no study has investigated whether the etiology of cirrhosis (alcohol-related or non-alcohol-related) has an impact on the rate of variceal hemorrhage. A secondary aim of the study was therefore to determine if the etiology of cirrhosis (alcohol-related *vs* non-alcohol related) correlated with rates of variceal hemorrhage in this era.

MATERIALS AND METHODS

This was a retrospective, cross-sectional review for all hospital admissions for gastroesophageal variceal hemorrhage to an intensive care unit at a tertiary referral center (University of Vermont/Fletcher Allen Health Care) between 1998 and 2009. Patients were initially identified based on International Classification of Disease (ICD-9) codes for upper gastrointestinal bleeding: esophageal varices with bleeding (456.0); esophageal varices in disease classified elsewhere with bleeding (456.20); hematemesis (578.0); blood in stool/melena (578.1) and hemorrhage of gastrointestinal tract, unspecified (578.9).

Bleeding secondary to variceal hemorrhage was confirmed by review of the inpatient hospital record and endoscopy database. Bleeding was attributed to varices if one of the following criteria was met: (1) actively bleeding varices visualized on endoscopy; (2) varices identified on endoscopy with stigmata of recent hemorrhage; or (3) clinical presentation consistent with upper gastrointestinal bleed (melena or hematemesis) and presence of varices on endoscopy with no other etiology for bleeding identified. Eligibility criteria for further inclusion were a diagnosis of cirrhosis and age at least 18 years.

Demographic and outcomes data were analyzed for the entire cohort, and subgroup analysis was performed according to the etiology of cirrhosis (alcoholic versus non-alcoholic). Patients admitted with their first variceal bleed were defined as "Index Bleeds". Patients admitted with their second or greater episode of variceal bleeding were defined as "Rebleeds". A model of end stage liver disease (MELD) score was calculated[13], where possible, when same day laboratory data were available.

During the same time period, data were collected on total hospital admissions per year and cirrhosis-related admissions. Cirrhosis-related admissions were categorized further by etiology using ICD-9 hospital billing codes: alcoholic cirrhosis of liver (571.2) and cirrhosis of liver NOS (571.5). Patients younger than 18 years old were excluded.

Variceal bleeding data were then directly compared to cirrhosis-related and total admission data and evaluated for trends.

Data on placement of transjugular intrahepatic portosystemic shunt (TIPS) were obtained using procedure volumes reports generated using the radiology information system for exam codes: SPTIPS, SPHEPATVEN and SPHEPATVNWO.

Statistical analysis

Using Pearson's χ^2 test for two proportions, we calculated that a sample size of $n = 129$ would result in 90% statistical power to detect a 15% change in variceal bleed % (at an alpha level of 0.05).

For analyses of linear trends over time for numeric

Table 1 Baseline characteristics of study population based on etiology of cirrhosis *n* (%)

	Overall	ETOH	Non-ETOH	*P* value
No. ICU admissions	243	151	92	
Mean age	54.33	52.92	58.25	0.002
Male sex	165 (67.9)	119	46	0.003
White ethnicity	235 (96.7)	147	88	0.04
Nonselective beta-blocker	82 (33.7)	45	37	0.51
Index bleed	128 (52.6)	78	50	0.07
Deaths	43 (17.6)	22	21	0.88

ETOH denotes alcoholic cirrhosis; Non-ETOH denotes non-alcoholic cirrhosis. ICU: Intensive care unit.

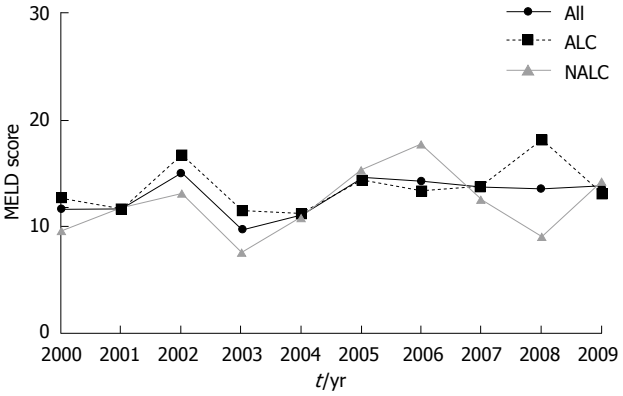

Figure 1 Mean model of end stage liver disease scores for study population per year. *P* value = 0.20 reflects trend for MELD scores for cirrhosis of all causes. *P* value = 0.27 reflects comparison of ALC and NALC groups. MELD: Model for end-stage liver disease; All: Cirrhosis all causes; ALC: Alcoholic cirrhosis; NALC: Non-alcoholic cirrhosis.

variables, linear regression analysis was employed to calculate Pearson correlation coefficients, and to determine statistical significance of trends. A "*P* value" of less than 0.05 was deemed statistically significant.

For analyses of linear trends over time for categorical variables (percentage positive responses of total for dichotomous variables), logistic regression analysis and/or analysis of two-way contingency tables (for Cochran-Mantel-Haenszel nonzero correlation statistics) were done. Using these methods, statistical significance of linear trends were determined, along with corresponding odds rates. A "*P* value" of less than 0.05 was deemed statistically significant.

RESULTS

Study population

A total of 1719 inpatient admissions were identified with upper gastrointestinal bleeding based on hospital discharge diagnosis. After review of the inpatient records, 243 admissions satisfied the criteria for bleeding attributable to gastroesophageal varices for the period 1998-2009 (Table 1). Approximately half of these were index bleeds. The mean age was 54.33 years, approximately two thirds of patients were male, and nearly all were white. Cirrhosis was attributed to alcohol in 62% of the patients. When

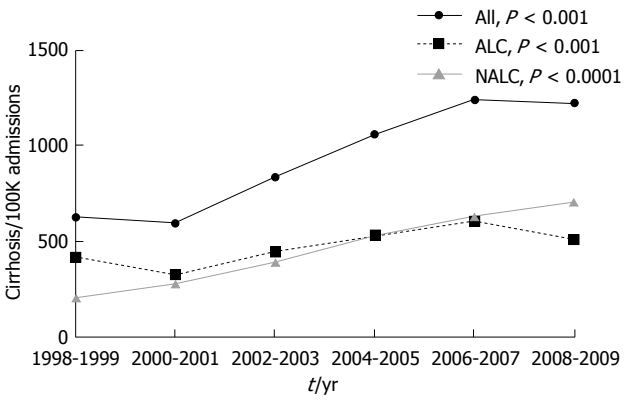

Figure 2 Admissions with cirrhosis per 100000 IP visits based on etiology of cirrhosis. *P* values are for trend. All: Cirrhosis all causes; ALC: Alcoholic cirrhosis; NALC: Non-alcoholic cirrhosis.

compared to non-alcoholic cirrhotics, the patients with alcohol-related variceal bleeding were significantly younger and more likely to be male. The average MELD score could be calculated for the years 2000-2009 and did not statistically change during this period nor did the average MELD for the cohort change over time (*P* value for trend = 0.20, Pearson correlation coefficient 0.44) (Figure 1). The average MELD score did not differ statistically between the two groups (*P* = 0.27).

Hospitalizations for cirrhosis

During the study period, total admissions for cirrhosis increased by more than 100% (Figure 2), from 611 per 100000 admissions in 1998-2001 to 1232 per 100000 admissions in 2006-2009 (*P* value for trend ⩽ 0.0001). Increases were observed regardless of etiology of cirrhosis. Admissions for alcoholic cirrhosis increased 52% during the study period, from 369 per 100000 admissions in 1998-2001 to 560 per 100000 admissions in 2006-2009 (*P* value for trend ⩽ 0.001). Admissions for non-alcoholic cirrhosis increased 175%, from 244 per 100000 admissions in 1998-2001 to 672 per 100000 admissions in 2006-2009 (*P* value for trend ⩽ 0.0001).

Hospitalizations for variceal hemorrhage

As shown in Figure 3A, the overall hospitalization rate for variceal bleeding decreased significantly from 96.6 per 100000 admissions in 1998-2001 to 70.6 per 100000 admissions from 2006-2009 (*P* value for trend = 0.01). When analyzed according to etiology, the hospitalization rate for variceal hemorrhage related to alcoholic cirrhosis did not statistically decrease during the study period (55 per 100000 admissions in 1998-2001, 50.9 per 100000 admissions in 2006-2009; *P* value for trend = 0.297). By contrast, hospitalization for variceal hemorrhage from non-alcoholic cirrhosis decreased significantly from 41.6 per 100000 admissions in 1998-2001 to 19.7 per 100000 admissions in 2006-2009 (*P* value for trend = 0.007).

As shown in Figure 3B, hospitalizations for index bleeding did not significantly change in either group (alcoholic cirrhosis: 23.48 per 100000 admissions in 1998-2001 to 30.91 per 100000 admissions in 2006-2009,

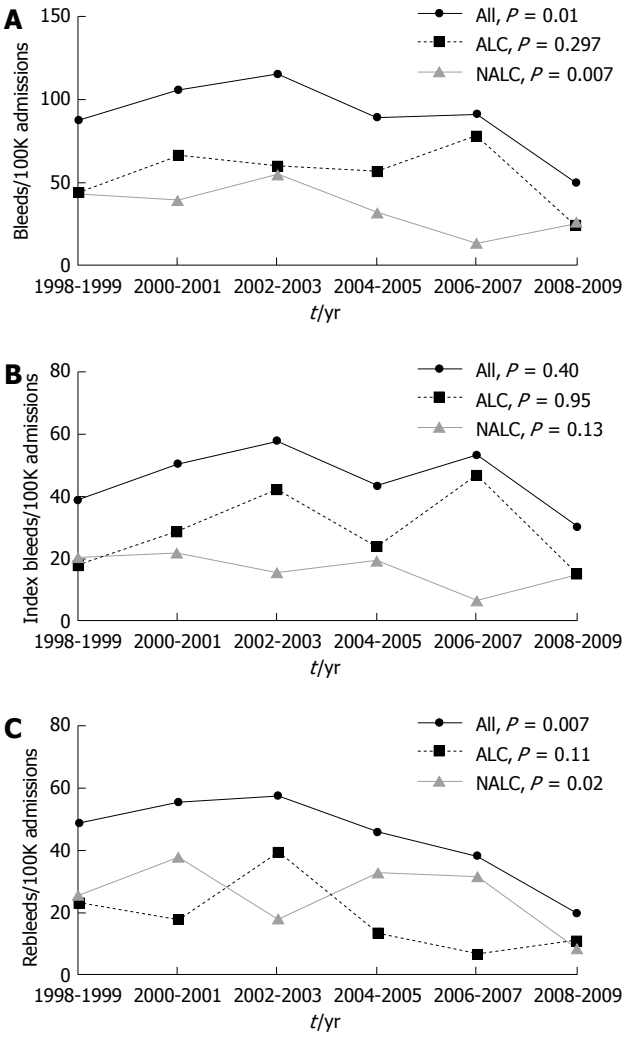

Figure 3 Trends in admissions for variceal bleeding per 100000 inpatient visits based on etiology of cirrhosis. A: Total variceal bleeds; B: Index variceal bleeds; C: Variceal rebleeding. *P* values are for trend during study period. All: Cirrhosis all causes; ALC: Alcoholic cirrhosis; NALC: Non-alcoholic cirrhosis.

P value for trend = 0.95, OR = 1.00; non-alcoholic cirrhosis: 29.14 per 100000 admissions in 1998-2001 to 10.92 per 100000 admissions in 2006-2009, *P* value for trend = 0.13, OR = 0.94).

Admission trends differed when there was a history of previous variceal hemorrhage. Hospitalizations for variceal rebleeding decreased for the overall study population (Figure 3C), from 51.85 per 100000 admissions in 1998-2001 to 28.72 per 100000 admissions in 2006-2009 (*P* value for trend = 0.007, OR = 0.93). This decrease remained significant after controlling for age (*P* value for trend = 0.01, OR = 0.93), and was seen specifically among patients with non-alcoholic cirrhosis (*P* value for trend = 0.02, OR = 0.91) but not in the alcoholic cirrhosis group (*P* value for trend = 0.11, OR = 0.95).

In parallel with the increased number of hospitalizations for cirrhosis, there was a significant increase in the number TIPS placements (Figure 4, *P* value for trend = 0.0006, Pearson correlation coefficient 0.84). A total of 101 TIPS were placed for all indications between 1998

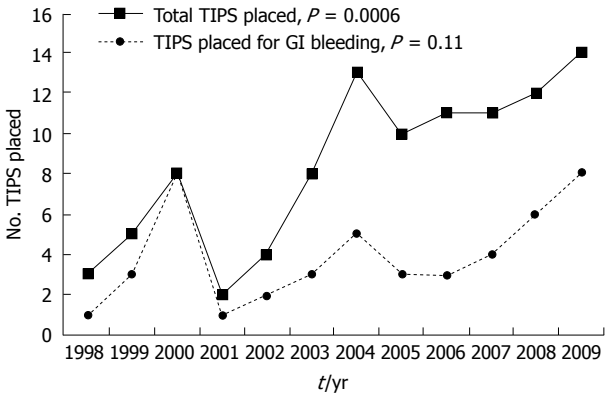

Figure 4 Transjugular intrahepatic portosystemic shunt placement 1998-2009. *P* values are for trend. TIPS: Transjugular intrahepatic portosystemic shunt; GI: Gastrointestinal.

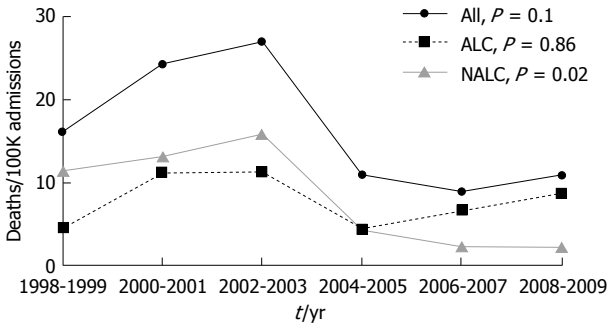

Figure 5 Hospital deaths per 100000 admissions based on etiology of cirrhosis. *P* value is for trend. All: Cirrhosis all causes; ALC: Alcoholic cirrhosis; NALC: Non-alcoholic cirrhosis.

to 2009, but the minority (47) were placed for portal hypertensive gastrointestinal bleeding. As can be shown in Figure 4, the number of TIPS placed for this indication fluctuated over the study period, but did not statistically significantly increase (*P* value for trend = 0.11, Pearson correlation coefficient 0.48).

Outcomes for variceal hemorrhage

Hospital deaths associated with variceal bleeding decreased significantly during the study period (Figure 5), from 20.09 per 100000 admissions in 1998-2001 to 9.83 per 100000 admissions in 2006-2009 (*P* value for trend = 0.04, OR = 0.914). This decrease was not statistically significant after controlling for age (*P* value for trend = 0.1). However, when controlling for age, death from variceal bleeding in the non-alcoholic cirrhotic group significantly decreased from 12.26 per 100000 admissions in 1998-2001 to 2.18 per 100000 admissions in 2006-2009 (*P* value for trend = 0.02, OR = 0.83); by contrast variceal hemorrhage-related death in the group with alcoholic cirrhosis did not significantly change (7.83 per 100000 admissions in 1998-2001 to 7.65 per 100000 admissions in 2006-2009, *P* value for trend = 0.86, OR = 0.99). Length of stay decreased significantly for the alcoholic cirrhosis bleeds (10.47 d in 1998-2001 to 5.71 d in 2006-2009), but not for the non-alcoholic cirrhosis bleeds (10.45 d in

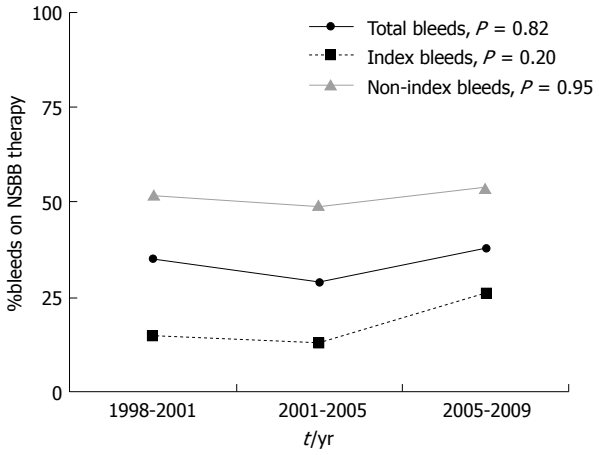

Figure 6 Non-selective beta-blocker usage amongst patients with variceal index bleeding and recurrent variceal bleeding. NSBB: Nonselective beta-blocker. *P* values are for trend.

1998-2001 to 7.39 d in 2006-2009), or the overall study population (10.12 d in 1998-2001 to 6.32 d in 2006-2009) (*P* value for trend = 0.03, 0.48, and 0.13; Pearson correlation coefficient = -0.062, -0.23, and -0.45 respectively).

Non-selective beta-blocker therapy

During the study period, 82 of 243 (33%) variceal bleeds occurred in patients taking non-selective beta-blockers (carvedilol, nadolol or propanolol)(NSBBs). Among patients with index bleeds, 17.7% were taking NSBBs, and this was significantly lower than the proportion of patients with re-bleeds (51.3%) who were taking NSBBs ($P < 0.0001$) (Figure 6). NSBB use did not change over time in the study cohort (35% of patients in 1998-2001 *vs* 38% patients in 2006-2009, *P* value for trend = 0.82). There was no significant trend in NSBB use over time in either the index-bleed or non-index bleed subgroups (*P* value for trend = 0.20 and 0.95 respectively).

DISCUSSION

This study has evaluated trends in hospitalizations to an intensive care unit for variceal bleeding during a period when pharmacological and endoscopic therapies for prevention of this problem were well-established and largely unchanged, as supported by published practice guidelines[12,14]. Our findings show that despite increasing hospitalization rates for cirrhosis of all causes, hospitalization rates for variceal bleeding have actually decreased, specifically in patients with cirrhosis from non-alcoholic etiologies.

Our observations extend those of previous work, which showed an increase in admissions from variceal bleeding from 10.9 to 12.4 per 100000 between 1988-1990 and 1994-1996, and then a decrease to 10.6 per 100000 in 2000-2002. During this entire period, strategies to prevent variceal hemorrhage were still evolving, and the prevalence of cirrhosis was increasing. Interactions between these two factors may have accounted for the changing incidence in hospitalizations. As shown in the present study, there has been a continued decrease in admissions for variceal bleeding, between 1998-2001 and 2006-2009, an era in which hospitalizations among cirrhotic individuals has continued to increase. The lack of an expected increase in variceal bleeding raises the possibility that enhanced application of prophylactic therapies may be offsetting the increasing burden of cirrhosis and thus the pool of patients at risk for bleeding from gastroesophageal varices. This is supported by published surveys, which showed increased adoption of such therapies by gastroenterologists in response to publication of practice guidelines[15]. Since our study did not show that index bleeding has decreased, it may be that these strategies have been mainly effective in the secondary prevention of variceal bleeding, at least among patients with non-alcoholic cirrhosis.

An alternative explanation for the reduction seen in variceal rebleeding could be increased placement of TIPS, which has evolved to become the standard of care in refractory variceal bleeding[14,16-21]. Whilst we observed an increase in TIPS placement over the study period, the overall number of TIPS placed was small in comparison to the cirrhotic population at risk for variceal hemorrhage. Thus, other factors are likely to be more important contributors to changing trends in hospitalizations for variceal bleeding.

There was a low rate of NSBB use in our cohort, particularly among those with index bleeds. Several factors may account for this. Among index bleeders, it is possible that not all patients underwent screening endoscopy prior to the episode of variceal bleeding, that some patients were intolerant of these agents, or that some did not adhere to medical recommendations. The significantly higher rate of NSBB use in rebleeding patients may be explained by more uniform adoption of consensus guidelines for secondary prophylaxis of variceal hemorrhage, but problems related to drug tolerance and patient adherence may have resulted in less than uniform drug usage. Our study was not designed to address these issues, but this would be appealing to assess in the future.

It is also notable that while admissions for variceal bleeding from non-alcohol-related variceal bleeding at our institution have significantly decreased, those from alcohol-related cirrhosis have been stable. One possible explanation for the difference in variceal bleeding rates between the two groups is that in patients with alcoholic cirrhosis, alcohol ingestion increases portal pressure[22], a major driving force for variceal hemorrhage. Alternatively, patients with alcoholic cirrhosis may not have similar access, in comparison with patient with nonalcoholic cirrhosis, to beneficial prophylactic measures. Since active alcoholism may negatively affect adherence to prescribed medications[23], it is possible that this may translate among patients with alcoholic cirrhosis into decreased utilization of measures to prevent variceal bleeding. This concept is further supported by our findings that rebleeding from varices has decreased only in non-alcoholic cirrhosis. Efforts to improve delivery and utilization of prophylactic therapies for variceal bleeding in patients with alcohol-related cirrhosis may result in similar reductions of bleeding.

A second major finding of this study is that in-hospital mortality associated with gastroesophageal variceal bleeding has decreased. This extends a decrease in mortality that was initially observed 30 years ago, which paralleled the emergence of robust targeted pharmacologic, endoscopic, and interventional radiologic treatments for control of variceal hemorrhage. Although such decreases were initially observed in clinical trials, they have been confirmed in larger populations, most recently in an analysis of a nationwide hospital database between 1988 and 2004[24]. It is striking that we have observed a continued decrease in mortality over a more recent time period, an era in which targeted therapies for variceal bleeding have not changed. The observed decrease in mortality is unlikely to be related to the prevalence of decompensated liver disease (a known risk factor for adverse outcomes in variceal hemorrhage[2]) in our patient cohort as the mean MELD score, an index of liver disease severity[13], did not change during the study period. It is tempting to speculate that the decrease in in-hospital mortality reflected changes in management not directly targeted to varices *per se*, such as administration of broad-spectrum antibiotics to prevent adverse sequelae (*e.g.*, spontaneous bacterial peritonitis, bacteremia) of variceal bleeding, which have been shown to decrease mortality[14,25]. Although this study was not designed to look at infectious outcomes, it is interesting to note that age-adjusted mortality decreases were seen in the non-alcoholic cirrhotic group but not in patients with a history of alcohol use, a known risk factor for increased mortality in critical illness accompanied by infections[26,27]. Whether the etiology of cirrhosis, and in particular, ongoing alcohol consumption influences the effectiveness of prophylactic antibiotics on morbidity and mortality of variceal hemorrhage is an intriguing question for future study.

Two additional points merit comment. First, because our study used ICD-9 coding data to identify patients, it is possible that patients hospitalized for variceal bleeding were missed by our analysis. However, this is unlikely, since the endoscopy database was examined in parallel, which allowed us to capture patients that would otherwise have been overlooked. Second, our study was performed in a single center in a predominantly rural region with limited ethnic diversity. As of 2010, 95.2% of the population of Vermont was reported as of white ethnicity[28]. Thus, it would be important to test whether our findings extend to other populations.

Collectively, our findings show that despite a large increase in admissions for cirrhosis for over a decade, hospitalizations and in-hospital mortality for variceal bleeding are decreasing, specifically in patients with non-alcoholic cirrhosis. This suggests that current management strategies for the prevention and treatment of variceal bleeding are having a positive impact on these outcomes. Since a decrease in hospitalizations was not observed for patients with index bleeds, this raises the possibility that cirrhosis was not recognized prior to the bleeding episode or that programs to diagnose and treat varices in asymptomatic cirrhotic patients have not been optimized. Improving access to preventative measures in such patients, and in particular, those with alcoholic cirrhosis, could potentially further reduce future hospitalizations for variceal hemorrhage.

COMMENTS

Background

The prevalence of cirrhosis, a consequence of long-term liver injury, has been increasing. Cirrhosis often leads to portal hypertension, and complications from portal hypertension, such as gastroesophageal variceal hemorrhage, result in significant morbidity and mortality. These adverse outcomes have been improved by prophylactic measures established well over 15 years ago, including endoscopic variceal ligation and non-selective beta-blockers, but treatment advances have been limited more recently. It is unknown how this limitation has influenced hospitalization trends during this more recent era.

Research frontiers

In analysis of tends for gastroesophageal variceal hemorrhage in an era in which preventative strategies were improving, prior work has shown that there has been a reduction in hospitalizations for this complication. In this study, the authors have sought to examine whether this trend has been maintained during an era in which no new preventative strategies have emerged, and whether such trends have been influenced by the etiology of cirrhosis.

Innovations and breakthroughs

In contrast to prior work, which analyzed trends for gastroesophageal variceal hemorrhage during an era of improving prophylactic strategies, the present study confines the analysis to a more recent era, in which such strategies have not improved further. The results show that hospitalizations for gastroesophageal bleeding have been decreasing, particularly in cirrhosis of non-alcoholic etiology. This raises the possibility that enhanced application of prophylactic therapies has offset the increasing burden of cirrhosis.

Applications

These findings suggest that in the absence of new prophylactic strategies against gastroesophageal bleeding, future reductions in hospitalizations for this complication of cirrhosis are likely to come from increased adherence to existing preventative programs. The authors suggest that new research be devoted to quality improvement in this area.

Terminology

Cirrhosis is a disorder, in response to chronic injury of extensive scar formation and distortion of blood flow within the liver. In cirrhosis, progressive resistance to liver blood flow increases pressure in the portal vein, the major blood vessel that supplies the liver. This condition, portal hypertension, can lead to several complications, including formation of gastroesophageal varices, engorged veins that are present beneath the interior surface of the esophagus and stomach. When the pressure in the portal vein increases above a critical value, bleeding from gastroesophageal varices can occur. Two strategies have been used successfully to prevent this problem, endoscopic techniques that directly apply elastic bands to obliterate esophageal varices, and medications (non-selective beta-blockers) that lower the pressure in the portal vein.

Peer review

This manuscript evaluates the hospitalization trends for variceal bleeding during a period when the prevalence of cirrhosis has increased. The study is well designed, all the section (from abstract to conclusions) are well written, and the topic is interesting for the readers.

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World J Gastroenterol 2014 August 28; 20(32): 11333-11339
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

RETROSPECTIVE STUDY

Epithelioid hemangioendothelioma of the liver as a rare indication for liver transplantation

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Received: December 27, 2013 Revised: February 25, 2014
Accepted: May 29, 2014
Published online: August 28, 2014

Abstract

AIM: To investigate the indications and outcomes of liver transplantation for hepatic epithelioid hemangioendothelioma (HEHE).

METHODS: Between 1989 and August 2013, in the Department of General, Transplant, and Liver Surgery, Medical University of Warsaw, 1306 orthotopic liver transplantations (OLTx) were performed, including 72 retransplantations. Unresectable HEHE was an indication for OLTx in 10 patients (0.8% of primary OLTx), the mean age of the patients was 40.5 ± 13.3 years (range 23-65 years), and the male-to-female ratio was 2:8. Kaplan-Meier survival analysis in HEHE, hepatocellular carcinoma (HCC), and other OLTx recipients groups was performed. The differences in mortality were compared using the χ^2 test. A P-value < 0.05 indicated statistical significance.

RESULTS: No concomitant liver disease was found in any patient. There was no neoadjuvant chemotherapy or radiotherapy. Liver function test results were normal in most of the patients. The levels of alpha-fetoprotein, carcinoembryonic antigen, and carbohydrate antigen 19-9 were normal. In immunohistochemical staining, the neoplastic cells were positive for factor Ⅷ-related antigen, CD31, and CD34, which are endothelial cell markers, and negative for cytokeratin 19, cytokeratin 7, and HepPar-1. Nine patients were alive without tumor recurrence. One patient died 2 mo after OLTx due to septic complications. No morbidity was observed. Maximum follow-up was 11.4 years, with a minimum of 1 mo. The cumulative survival rate at the end of follow-up in HEHE patients was 87.5% compared with 54.3% in the HCC group and 76.3% in the other OLTx recipients group (χ^2 test = 1.784, df = 2, P = 0.409).

CONCLUSION: Unresectable HEHE, without extrahepatic metastases is an excellent indication for liver transplantation. Long-term survival is very good and much better than in HCC patients and the entire group of OLTx patients.

Key words: Hemangioendothelioma; Liver transplantation; Liver malignancies; Transplantation results; Transplantation indications

Core tip: Epithelioid hemangioendothelioma (EHE) of the liver (hepatic EHE, HEHE) is a very rare tumor of mesenchymal origin. It typically occurs in female patients aged 20-40 years. HEHE is resistant to chemotherapy. Unresectable tumor, limited to the liver, may be a good indication for liver transplantation. The aim of this paper was to analyze the indications and outcomes of liver transplantation for HEHE.

Remiszewski P, Szczerba E, Kalinowski P, Gierej B, Dudek K, Grodzicki M, Kotulski M, Paluszkiewicz R, Patkowski W, Zieniewicz K, Krawczyk M. Epithelioid hemangioendothelioma of the liver as a rare indication for liver transplantation. *World J Gastroenterol* 2014; 20(32): 11333-11339 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11333.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11333

INTRODUCTION

Hemangioendothelioma (epithelioid hemangioendothelioma, EHE) is a rare tumor of endothelial and connective tissue origin, resembling hemangioma. This type of tumor was first described by Weiss and Enzinger[1]. Its incidence does not exceed 1 case per million[2]. EHEs are found in soft tissue and internal organs. The most commonly affected organ is the liver (hepatic EHE, HEHE). Other localizations such as the lungs, peritoneum, spleen, bones, brain, meninges, breast, heart, head, and stomach have been described in the literature[3-7].

The mechanisms underlying the development of HEHE are not known. Possible risk factors include oral contraception, toxicity associated with vinyl chloride or asbestos exposure, excessive alcohol consumption, liver injuries, viral hepatitis, and other chronic liver diseases[8].

Numerous recent studies also suggest genetic mechanisms underlying the development of EHE. Specific chromosomal translocation t(1;3)(p36.3;q25) is typical for this type of tumor[9-11].

HEHE is typically diagnosed in young females aged 20-40 years. The prognosis without treatment is poor. Most patients present with disseminated disease, which usually involves both lobes of the liver. Currently there are several methods of treatment available, including surgery (liver resection and transplantation), chemotherapy, transarterial chemoembolization (TACE), radiotherapy, and radiofrequency ablation (RFA). Multifocal unresectable HEHE may be an indication for OLTx[12].

The aim of this study was to assess the outcomes of OLTx in patients with HEHE.

MATERIALS AND METHODS

Between 1989 and August 2013, 1306 orthotopic liver transplantations (OLTx) in adults including 72 re-transplantations (re-OLTx) were performed in the Department of General, Transplant, and Liver Surgery, Medical University of Warsaw. Unresectable HEHE was an indication for OLTx in 10 patients. The mean age of patients was 40.5 ± 13.3 years (range 23-65 years), and the male-to-female was ratio 2:8.

Statistical analysis

We compared Kaplan-Meier distributions of the time to death between HEHE and hepatocellular carcinoma (HCC) groups and with the general liver transplant population, using the χ^2 test. The differences were considered statistically significant when the *P*-value was < 0.05.

RESULTS

The initial referral diagnosis was usually different from the final diagnosis of HEHE. Most commonly the lesions were described as hemangioma, metastases of unknown origin, or parasitic abscesses. In our Department, the diagnosis was based on a wedge liver biopsy obtained during diagnostic laparoscopy. In 2 patients the diagnosis was based on radiologic assessment of the lesions in the liver (computed tomography or magnetic resonance imaging). No patient had neoadjuvant chemotherapy or radiotherapy. Liver function test results were normal in most of the patients. In 4 more advanced cases mild elevation of markers of cholestasis such as alkaline phosphatase (ALP) and gamma-glutamyl transpeptidase (GGTP) was observed. In all the patients with HEHE, the levels of alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA), and carbohydrate antigen 19-9 (CA19-9) were normal (Table 1).

No concomitant liver disease was found in any patients. The symptoms were usually nonspecific, with mild epigastric or right upper quadrant pain or discomfort. Sometimes fatigue or weight loss was reported (Table 2). The severity of the disease symptoms increase as the disease progressed, with increasing numbers and size of the lesions.

Macroscopically, cross-sections of the resected liver showed multifocal non-encapsulated, yellowish-white tumors that involved both lobes, ranging in diameter from 0.2 to 18 cm. Subcapsular tumors showed typical umbilication.

Microscopically, the tumors had a high cellular growth pattern with infiltrative margins. Histopathological examination demonstrated a variable cellularity - the cellular components were dominant in the peripheral region, while central areas of the nodules showed both necrosis and sclerosis and some were calcified. In the more cellular areas, tumor cells were arranged in solid cords and nests, mimicking epithelioid or histiocytoid cells. In other, less cellular, areas having a sclerotic matrix, single neoplastic cells had irregular cytoplasmic processes. Epithelioid cells were round in shape and had an eosinophilic abundant cytoplasm and vesicular nuclei. Dendritic cells displayed spindle morphology with interdigitating processes. Characteristically, tumors consisted of spindle-shaped cells and signet ring cell-like structures with intracytoplasmic lumina that contained erythrocytes. Architectural features, such as an intravascular or intrasinusoidal growth pattern characteristic of a neoplasm were observed. Microvascular invasion was found in 2 cases. The parenchymal architecture of the liver was preserved. In immunohistochemical staining, the neoplastic cells were positive for factor Ⅷ-related antigen, endothelial cell markers CD31 and CD34, and negative for cytokeratin 19, cytokeratin 7, and HepPar-1 (Figure 1 and Table 3).

The follow-up for the HEHE group ranged from 1 mo to 11.4 years. There was no tumor-related mortality.

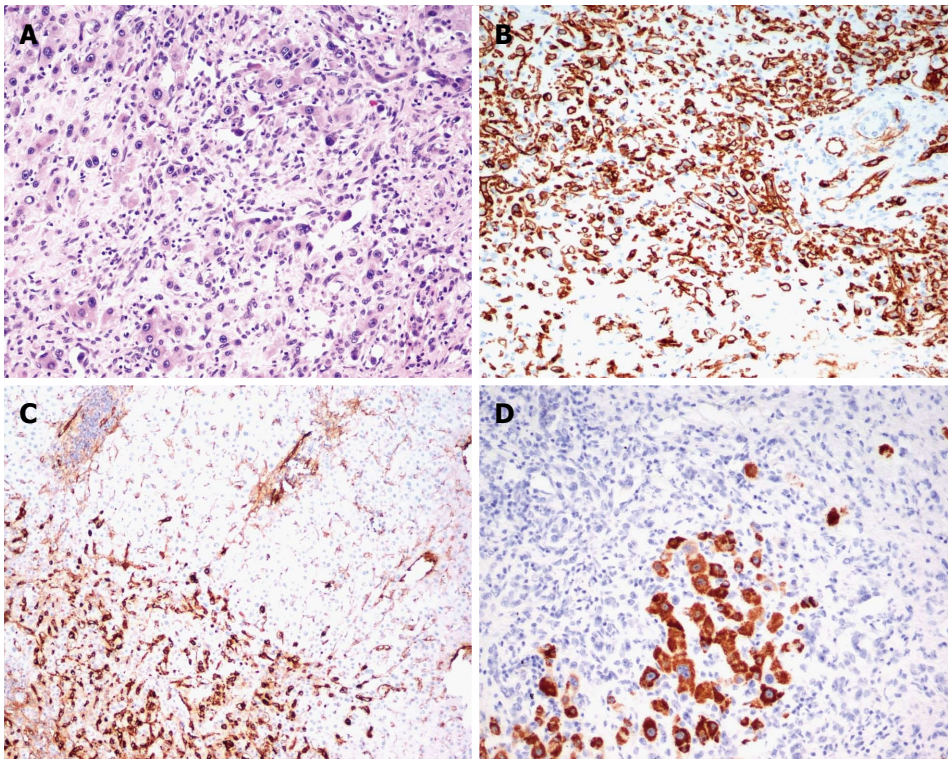

Figure 1 Hepatic epithelioid hemangioendothelioma microscopic staining. A: Hematoxylin and eosin staining × 20; B: CD34 staining × 20; C: Factor Ⅷ staining × 10; D: HepPar1 staining × 20.

Table 1 Patient data before orthotopic liver transplantations

Patient	AST (U/L)	ALT (U/L)	Bilirubin (mg/dL)	Albumin (g/dL)	GGTP (U/L)	ALP (U/L)	Creatinine (mg/dL)	Urea (mg/dL)	Fibrinogen (mg/dL)	PLT (10^3/μL)	INR	CEA (ng/mL)	CA 19.9 (IU/mL)	AFP (ng/mL)
1	36	112	1.36	4.20	606	NA	0.88	48.0	NA	314	NA	NA	NA	NA
2	27	36	0.64	3.90	178	206	0.70	17.8	539	291	1.11	0.98	2.73	4.48
3	155	141	0.50	3.40	439	185	0.76	35.8	368	249	NA	1.49	2.00	2.01
4	19	20	0.76	4.70	35	81	0.63	19.0	236	150	0.96	0.8	7.70	1.6
5	13	24	0.71	4.60	28	74	0.99	47.0	250	203	1.01	1	0.70	1.3
6	96	121	1.33	4.40	13	47	0.75	20.0	412	207	1.10	1.2	22.90	1.5
7	56	86	0.67	3.29	297	331	0.69	24.0	332	199	1.01	0.8	18.80	1.9
8	24	28	0.69	4.03	19	79	0.73	25.0	468	42	1.02	1	4.50	1.1
9	26	30	0.85	4.14	78	87	0.89	25.0	356	100	1.10	5.9	12.30	3.1
10	33	32	0.61	3.50	321	329	0.98	37.4	593	290	0.93	0.71	25.80	2.2

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGTP: Gamma-glutamyl transpeptidase; ALP: Alkaline phosphatase; AFP: Alpha-fetoprotein; CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; PLT: Platelets; INR: International normalized ratio; NA: Not avaible.

Table 2 Patient symptoms

Patient	Symptoms
1	NA
2	NA
3	NA
4	Abdominal pain or discomfort; fatigue
5	None
6	NA
7	Abdominal pain or discomfort
8	NA
9	Abdominal pain or discomfort; fatigue; weight loss; hepatomegaly; dyspnea
10	Abdominal pain or discomfort

NA: Not available.

One patient died 2 mo after OLTx due to septic complications not related to the nature of the HEHE. When this case of tumor-unrelated death was excluded from the analysis, the long-term survival in HEHE patients was 100%. No morbidity was observed. The remaining 9 patients are alive without any tumor recurrence symptoms.

Among all patients after OLTx (n = 1234), 3 groups were defined for the purpose of survival analysis: patients with HEHE (n = 10); patients with HCC (n = 155), which is the most common tumor considered an indication for OLTx; and the remaining group of OLTx recipients (n = 1069).

Kaplan-Meier analysis showed a 3-year cumulative survival rate of 87.5% for the HEHE group, 80.1% for

Table 3 Histopathological and immunological characteristics of the tumors

Patient	Lymph node metastases	Lesions (*n*) max diameter	Carcinoma cells microembolisms	CD31	CD34	Factor Ⅷ	CK7	MiB	PCNA	Hep	CK19
1	+	Multi focal 60 mm	+	+	+	+	-	-	+	-	NA
2	-	Multi focal 180 mm	NA	NA	+	+	-	NA	NA	-	NA
3	+	Multi focal 55 mm	+	NA	+	NA	-	NA	NA	-	-
4	NA	6 tumors 27 mm	NA	+	+	NA	NA	NA	NA	NA	NA
5	-	9 tumors 30 mm	NA	+	+	+/-	-	NA	NA	-	NA
6	NA	2 tumors 30 mm	NA	NA	+	+	NA	NA	NA	NA	NA
7	-	Multi focal 70 mm	NA	+	+	NA	NA	NA	NA	-	-
8	-	Multi focal 30 mm	NA	NA	+	NA	NA	NA	NA	NA	NA
9	NA	Multi focal 40 mm	NA	+	+	+	NA	NA	NA	-	-
10	-	Multi focal 20 mm	NA	+	+	+	NA	NA	NA	NA	-

NA: Not available.

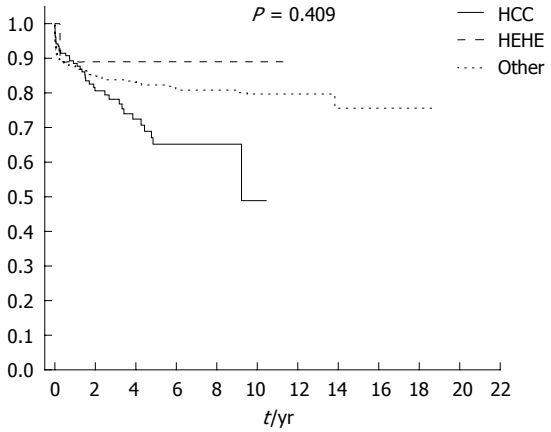

Figure 2 Kaplan-Meier survival analysis. Kaplan-Meier survival analysis in the hepatic epithelioid hemangioendothelioma (HEHE), hepatocellular carcinoma (HCC), and other orthotopic liver transplantations recipients groups.

the HCC group, and 83.5% in the remaining OLTx recipients group.

The cumulative survival rate at the end of the follow-up was 87.5% in the HEHE patients, compared with 54.3% in the HCC group and 76.3% in the other OLTx recipients group (χ^2 test = 1.784, df = 2, P = 0.409) (Figure 2).

DISCUSSION

EHE is a very rare tumor. The low incidence precludes any randomized clinical study for the assessment of the best means of treatment. The available publications usually present a retrospective assessment of a single center experience and most of the papers are single case reports. The prognosis depends on the organ involved and, for a hepatic localization, the overall outcome is poor. The disease follows an unpredictable course, which is another confounding factor in the development of a treatment strategy. Disease progression may vary widely from mild with long-term survival[13,14] to severe with rapid deterioration[15,16]. Cases of spontaneous complete remission of HEHE have also been published[17]. There are no reliable predictive factors allowing for the assessment of prognosis. Well known tumor-related factors such as mitotic index, allowing for prediction of malignant potential, did not show any predictive value in cases of HEHE and are considered of low value in clinical practice.

In most of the cases, the disease is asymptomatic or the symptoms are only mild. The most commonly reported symptoms include abdominal pain or right subcostal discomfort related to hepatomegaly. Generalized cachexia or jaundice is less commonly present[18]. Similar symptoms were also observed in the current study patients. Most of the patients presented with normal blood tests. There were increased ALP and GGTP levels present in some cases but the levels of tumor markers such as CEA, CA19-9, and AFP were normal. These results are typical for HEHE[19].

All the studied HEHE tumors had typical pathology and immunohistochemical pattern with positive factor Ⅷ staining and positive endothelial markers CD31 (platelet endothelial cell adhesion molecular 1) and CD34 (human hematopoietic progenitor cell antigen)[20,21].

In 2006, Mehrabi *et al*[22] published a meta-analysis of studies published between 1984 and 2005 that included 402 cases of HEHE. Treatment details and outcomes were available for 286 cases. It was the largest meta-analysis of HEHE studies. At the time of diagnosis, most patients presented with symptoms of the disease, including, most commonly, right epigastric pain, hepatomegaly, and weight loss. Most of the patients had a multifocal involvement of both lobes of the liver. Extrahepatic disease was present in 36.6% of the patients. The most commonly encountered extrahepatic localizations were the lungs (8.5%), lymph nodes (7.7%), peritoneum (6.1%), bones (4.9%), spleen (3.2%), and diaphragm (1.6%). The most common method of treatment according to the meta-analysis was OLTx (44.8% of cases). Limited extrahepatic focal lesions were not considered an absolute contraindication to OLTx. The outcomes of OLTx are shown in Table 3. In the meta-analysis, 1-year disease-free survival was 81.3% (Table 4). The disease recurrence rate was 27% regardless of the treatment method used. Most of the HEHE liver recurrence was observed more than 2 years after transplantation. There was no recurrence in the study group. In the meta-analysis of Mehrabi *et al*[22], 21% of patients received radiotherapy or systemic chemotherapy or TACE. There is no consensus on pref-

Table 4 Hepatic epithelioid hemangioendothelioma treatment results summary

Ref.	Year (analyzed period)	Source	Patients		Survival percentage					
					OLTx	Liver resection	Chemio radio therapy	TACE	No treatment	All
Recent report	2013 (1989-2013)	Single center	10	3-yr	87.5%	-	-	-	-	-
Rodrigue *et al*[25]	2008	UNOS	110	1-yr	80%	-	-	-	-	-
				3-yr	68%					
				5-yr	64%					
Lerut *et al*[24]	2007	ELTR	59	1-yr	93%	-	-	-	-	-
				5-yr	83%					
				10-yr	72%					
Mehrabi *et al*[22]	2006 (1984-2005)	Meta-analysis	286	1-yr	96%	100%	72%	-	40%	83.4%
				3-yr	80%	87%	49%		12%	55.8%
				5-yr	54.5%	75%	30%		4.5%	41.1%
Wang *et al*[26]	2012 (2004-2011)	Single center	33	3-yr	-	74.1%	-	81.6%	-	73.3%
Grotz *et al*[27]	2010 (1984-2007)	Single center	30	1-yr	91%	100%	57%	-	57%	-
				3-yr	73%	86%	43%		43%	
				5-yr	73%	86%	29%		29%	
				10-yr	42.2%	86%	-		-	
Nudo *et al*[28]	2008 (1991-2005)	Multi center	11	5-yr	82%	-	-	-	-	-

OLTx: Orthotopic liver transplantations; TACE: Transarterial chemoembolization; UNOS: United Network for Organ Sharing; ELTR: European Liver Transplant Registry.

erable treatment, no criteria for introducing specific medications into a treatment plan, and no prospective studies evaluating their efficacy. In non-randomized observational studies, several drugs such as thalidomide, doxorubicin, 5-fluorouracil, and vincristine led to a reduction in lesion size or improvement in general status. TACE seems to be an acceptable bridge treatment in patients awaiting liver transplantation, similar to patients with HCC. There are single reports of successful use of interferon alpha 2b in the treatment of extrahepatic disease. Limited experience with radiotherapy does not provide enough data to assess its clinical significance. Moreover, in most of the radiotherapy cases, this method was used in combination with chemotherapy. In patients receiving chemotherapy or radiotherapy, 5-year survival was 30%. Liver resection was performed in 9.4% of the patients in the meta-analysis of Mehrabi *et al*[22]. Radical liver resection might be a treatment of choice, but usually the disease is locally advanced at the time of diagnosis and the patients do not qualify for surgery. Palliative resection is not recommended for this type of tumor due to a high risk of progression and recurrence after surgery[23]. The prognosis after liver resection is independent of the presence of extrahepatic lesions; therefore, extrahepatic disease should not be considered a contraindication to surgical intervention. Mehrabi *et al*[22] reported that 25% of patients with HEHE did not receive any treatment at all. In most of these cases, 1-year survival was less than 50%. However, there are cases of spontaneous regression. The 5-year survival rate was less than 5%. The longest reported survival exceeded 27 years after the diagnosis of HEHE. Mehrabi *et al*[22] suggested an algorithm to guide selection of the best treatment for patients with HEHE. Histopathological confirmation of the tumor is followed by the assessment of surgical resectability and the presence of extrahepatic disease. Liver resection should be considered an option, if possible. In patients with bilobar intrahepatic tumor spread without an involvement of other viscera, liver transplantation is the treatment of choice. Adjuvant chemotherapy, neoadjuvant TACE, chemotherapy, and radiotherapy are all available treatment options for cases of extrahepatic lesions.

Lerut *et al*[24], published data from the European Liver Transplant Registry (ELTR) presenting outcomes of liver transplantation in 59 patients with HEHE and an average follow-up of 78.5 mo. One-third of patients underwent some type of intervention before OLTx. In 17% of patients, extrahepatic lesions were found before OLTx or during the transplantation. Intrahepatic dissemination was present in 96% of the patients. Survival data is shown in Table 3. Recurrence-free survival observed at 1 year, 5 years, and 10 years of follow-up was 90%, 82%, and 64%, respectively. Tumor recurrence was found in 23.7% of patients at an average of 49 mo after OLTx. Recurrence-related mortality was 15.3%. Vascular involvement had a significant influence on the long-term prognosis. Survival was not affected by lymph node metastases, prior treatment, nor the presence of extrahepatic disease[24].

Rodriguez *et al*[25], in a study based on the United Nations for Organ Sharing database, presented data of 110 patients after OLTx performed between 1987 and 2005. The authors reported survival only slightly inferior to the results from the ELTR database (Table 4)[25].

Wang *et al*[26] reported the outcomes of 33 patients with HEHE who underwent various interventions other than OLTx. Liver resection was performed in 17 patients, TACE in 12 patients, and liver resection followed by TACE in 3 patients. Only 1 patient had OLTx performed in this group. There was no significant difference in 3-year survival between the treatment groups (Table 4). The authors selected several factors influencing treatment outcome. Older age, clinical symptoms, and elevated CA 19-9 were associated with poorer survival rates. The pa-

tients with symptoms (n = 17; P = 0.001, hazard ratio = 86.5) (12 with abdominal pain or discomfort, 3 with chest pain, 1 with weight loss, and 1 with jaundice) had poorer overall survival. The presence of symptoms was validated as the only significant independent prognostic factor (P = 0.012) by multivariate analysis[26].

Grotz *et al*[27] presented outcomes of 30 patients with HEHE who received treatment at the Mayo Clinic between 1984 and 2007. Liver resection was performed in 11 patients, OLTx in 11 patients, chemotherapy in 5 patients, and the remaining 3 patients received no intervention. The survival rates were similar to the studies reported previously (Table 4). The authors suggested that liver resection may be a suitable solution in patients with a solitary lesion limited to the liver, and OLTx may be offered to patients with multiple lesions in the liver (> 10 lesions and involvement of > 4 segments). Both methods resulted in comparable outcomes regarding survival and recurrence rate. According to the authors from the Mayo Clinic, the extrahepatic lesions (present in 37% of the patients) should not be considered a contraindication to surgery, because they did not influence the outcomes in the study group. Chemotherapy was not effective in this group of patients. Grotz *et al*[27] presented a treatment protocol based on selected risk factors. The authors focused on the type of intrahepatic dissemination of the tumor, the number of segments involved, the number and size of the lesions, and the presence of extrahepatic disease. Liver resection or RFA of the tumors was suggested in cases with a maximum of 10 lesions measuring not more than 10 cm in diameter and involving not more than 4 segments of the liver. In the other cases, OLTx was recommended. Extrahepatic lesions were not considered a contraindication to any form of surgical intervention, but chemotherapy and metastasectomy were strongly suggested. The authors also recommended liver resection or RFA in the case of local recurrence in the liver[27].

Nudo *et al*[28], in a Canadian multicenter study, presented treatment outcomes of 11 patients. Four patients received adjuvant therapy for HEHE before OLTx [interferon therapy (n = 1), splenectomy (n = 1), adriamycin therapy (n = 1), and surgical resection (n = 1)]. There was a 36.4% recurrence rate of HEHE during follow-up (on average, 25 mo from OLTx). Two patients with local recurrence underwent liver resection. In the 2 remaining cases of recurrence, radiotherapy or pegylated interferon was administered[28].

Cardinal *et al*[29] analyzed results of 25 patients who received treatment during 1976-2007 and found that the mean survival time was longer in the OLTx group (172 mo) compared with the TACE group (83 mo), but this difference did not reach statistical significance.

Liver resection should be considered the treatment of choice in patients with resectable HEHE. However, a substantial group of patients present with locally advanced disease that is initially unresectable. The results of the present study show that OLTx is a valid and effective method of treatment in patients with unresectable HEHE. The survival rates of patients after OLTx for HEHE were superior to survival rates of patients with HCC who underwent OLTx. Moreover, the survival of HEHE patients was better than the survival of patients with OLTx for other indications. The small number of patients in the HEHE group compared with the other groups of patients has to be considered, since it may lead to a significant statistical bias. The outcomes presented in the current study are comparable to the best outcomes published in the literature. The authors of the current study suggest following a reasonable qualification protocol that enables selection of an appropriate treatment for a specific patient. In the study group, all of the patients presented with the disease limited to the liver, which might positively influence the outcomes, but most of the authors do not consider extrahepatic lesions a contraindication to OLTx. The use of multiple methods of treatment may result in some benefits for survival related to the presumed synergistic effect of combined surgery and chemotherapy, radiotherapy, or TACE. Further studies are necessary to refine treatment protocols in HEHE, but it may be impossible to produce good quality evidence due to the very low incidence of the tumor.

COMMENTS

Background

Epithelioid hemangioendothelioma (EHE) is a rare tumor of endothelial and connective tissue origin. EHEs are found in soft tissue and internal organs. The most commonly affected organ is the liver. Hepatic EHE (HEHE) is typically diagnosed in young females aged 20-40 years. Most patients present with disseminated disease, which usually involves both lobes of the liver. The treatment options include surgery (liver resection and transplantation), chemotherapy, transarterial chemoembolization (TACE), radiotherapy, and radiofrequency ablation. Multifocal unresectable HEHE may be an indication for orthotopic liver transplantation (OLTx).

Research frontiers

Adequate selection for OLTx is of utmost importance among patients with liver tumors. The comparison of outcomes in OLTx for HEHE with outcomes in patients with other tumors, especially hepatocellular carcinoma, provides a good basis for decision-making in this type of indication for transplantation.

Applications

The results of the present study show that OLTx is a valid and effective method of treatment in patients with unresectable HEHE. A reasonable qualification protocol that enables selection of an appropriate treatment for a specific patient is needed. The use of multiple methods of treatment may result in some benefits for survival related to the presumed synergistic effect of combined surgery and chemotherapy, radiotherapy, or TACE. Further studies are necessary to refine treatment protocols in HEHE but it may be impossible to produce good quality evidence due to the very low incidence of the tumor.

Peer review

The authors described a series of 10 HEHE cases that were candidates for liver transplantation. As reported by the authors, the outcome was very good compared to HCC and other liver diseases as a whole. In general, the article is interesting and represent the experience of a single center, adding a new experience to other published reports, which were well-discussed by the authors.

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P- Reviewer: Akarsu M, Galvao FHF, Sira MM, Rodriguez-Peralvarez M **S- Editor**: Gou SX
L- Editor: Cant MR **E- Editor**: Wang CH

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DOI: 10.3748/wjg.v20.i32.11340

World J Gastroenterol 2014 August 28; 20(32): 11340-11346
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

RETROSPECTIVE STUDY

Decreased expression of miR-133a correlates with poor prognosis in colorectal cancer patients

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Supported by Grants from the National Key Clinical Medical Specialties Foundation and the National Natural Science Foundation of China, No. 81271916 and No. 81301506

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Received: December 11, 2013 Revised: February 24, 2014
Accepted: May 23, 2014
Published online: August 28, 2014

Abstract

AIM: To investigate microRNA-133a (miR-133a) expression in colorectal cancer (CRC) and its relationship with tumorigenesis and disease prognosis.

METHODS: Quantitative real-time polymerase chain reaction was used to measure levels of miR-133a in tumor samples and adjacent non-cancerous tissues from 169 patients undergoing radical resection for CRC. The associations between miR-133a expression and patient age, sex, as well as clinicopathologic parameters, such as tumor size, differentiation, location, invasion depth, metastasis, tumor-node-metastasis (TNM) stage and overall patient survival, were analyzed by Mann-Whitney *U* and Kruskal-Wallis tests. The Kaplan-Meier method and Cox proportional hazards regression analyses were performed to estimate the prognostic factors for patient survival prediction.

RESULTS: The expression of miR-133a was significantly downregulated in CRC tissues compared with adjacent non-cancerous tissues ($P < 0.05$). This reduction was associated with the depth of the local invasion, poor differentiation, lymph node metastasis and advanced disease ($P < 0.05$). Moreover, Kaplan-Meier analysis demonstrated that patients with low miR-133a expression had poorer overall survival (OS) than those with high miR-133a expression ($P < 0.001$). Univariate analysis revealed statistically significant correlations between OS and miR-133a level, tumor local invasion, lymph node metastasis and TNM stage ($P < 0.001$). Furthermore, miR-133a levels and TNM stage were independently associated with OS (HR = 0.590, 95%CI: 0.350-0.995, $P < 0.05$; and HR = 6.111, 95%CI: 1.029-36.278, $P < 0.05$, respectively).

CONCLUSION: The downregulation of miR-133a may play an important role in the progression of CRC and can be used as an independent factor to determine CRC prognosis.

Key words: Colorectal cancer; Biomarker; MicroRNA-133a; Prognosis; Real-time polymerase chain reaction

Core tip: In the present study, the level of microRNA-133a (miR-133a) was found to be downregulated in colorectal cancer (CRC) tissues. The altered expression of miR-133a was significantly associated with malignant behavior, including tumor cell differentiation, local invasion, lymph node metastasis and tumor-node-metastasis stage. Multivariate analysis suggested that low expression of miR-133a is an independent prognostic factor for CRC. Furthermore, the data suggest that miR-133a may play a critical role in CRC progression, and thus may serve as a potential therapeutic target.

Wang LL, Du LT, Li J, Liu YM, Qu AL, Yang YM, Zhang X, Zheng GX, Wang CX. Decreased expression of miR-133a correlates with poor prognosis in colorectal cancer patients. *World J Gastroenterol* 2014; 20(32): 11340-11346 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11340.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11340

INTRODUCTION

Colorectal cancer (CRC) is the second most common malignancy in females, and third most common in males worldwide, with over 1.2 million new cases and an estimated 608700 deaths in 2008 alone[1]. The five-year survival rate of CRC ranges from 90% for stage I patients to 8% for metastatic cases, and nearly 25% of patients with stage Ⅱ may relapse or develop metastases[2]. Biomarkers such as carcinoembryonic antigen have been recommended for the prediction of CRC prognosis and postoperative surveillance in advanced disease[3], however, they show a limited sensitivity of only 30%-40% for early diagnosis and prognosis. Therefore, novel and reliable prognostic CRC biomarkers are urgently needed.

MicroRNAs (miRNAs) are a novel class of small endogenous non-coding RNAs (17-28 nucleotides) that are involved in the initiation and progression of tumors by the dysregulation of oncogenes and tumor suppressor genes. A growing amount of evidence demonstrates the valuable role of miRNAs in tumor diagnosis, progression and therapy response, which has led both researchers and clinicians to focus on the identification of novel miRNAs. Ma *et al*[4] carried out a comprehensive systematic review of miRNA expression in CRC and identified several up- and downregulated miRNAs as candidate biomarkers. Among those identified, the ectopic expression of miR-133a was associated with various human malignancies, including lung squamous cell carcinoma, breast cancer, renal cell carcinoma, prostate cancer and bladder urothelial carcinoma[5-9]. In addition, a correlation between miR-133a expression and the carcinogenesis of CRC has also been reported[10,11]. Recently, Wang *et al*[12] demonstrated that miR-133a affects CRC cell motility and represses tumor growth and metastasis by targeting Lin11, Isl-1 and Mec-3 and SRC homology 3 protein 1, and inhibiting the mitogen-activated protein kinase (MAPK) pathway. However, another study showed that miR-133a served as a gene promoter for brain metastasis[13]. Thus, further analyses are needed to clarify the role of miR-133a in CRC prognosis based on clinicopathologic stage. In the present study, miR-133a expression levels in CRC were examined, and the clinicopathologic significance and potential prognostic value for CRC were assessed.

MATERIALS AND METHODS

Patients and sample collection

A total of 182 CRC patients who underwent radical resection for CRC in the Department of General Surgery, Qilu Hospital of Shandong University between June 2005 and December 2007 were recruited for this study. Of these subjects, seven were excluded because of incomplete follow-up data and six with distant metastases were excluded for the reason of non-statistical significance. The remaining 169 patients had not received preoperative adjuvant therapy and were deemed eligible for the study. All patient data were obtained from clinical and pathologic records, including age, sex, tumor size and depth, lymph node metastasis and distant metastasis. The postoperative pathologic staging of each subject was determined according to the 7th edition of the Union for International Cancer Control tumor-node-metastasis (TNM) staging system for CRC. The resected tumor tissues and paired adjacent non-cancerous tissues (at least 5 cm away from the tumor margin) were immediately collected, frozen in liquid nitrogen and stored at -80 ℃. This study was approved by the ethics committee of the Qilu Hospital of Shandong University and written informed consent was obtained from each patient or legal representative.

Follow-up

The patients were followed every 3 to 6 mo after the operation. The clinical end point of this study was death or the end of the study period (January 2013) with a median follow-up period of 63 mo (range: 10-77 mo). Overall survival (OS) was defined as the period from surgery to death. All data including physical examination, laboratory results and computed tomography findings were collected from hospital records or by patient interviews.

Cell culture

The human colon cancer cell line Caco-2 was kindly provided by Shuo Chen (Department of Gastroenterology, Qilu Hospital, Shandong University, China). Cells were cultured in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, United States), supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, United States) and maintained at 37 ℃ with 5% CO_2.

RNA preparation and quantitative real-time polymerase chain reaction

Total RNA was isolated from tissue or cells using TRIzol® reagent (Invitrogen, Carlsbad, CA, United States) following the manufacturer's instructions and the concentration was determined using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, United States). The cDNA was synthesized as follows: (1) 1 μg of isolated RNA was incubated at 65 ℃ for 5 min with 1 μL specific reverse transcription primer (RiboBio, Guangzhou, China), 1 μL U6 reverse transcription primer, and 1 μL dNTP in a 12 μL total reaction volume; (2) 4 μL of 5 × first-strand buffer, 2 μL of DTT, and 1 μL of RNase inhibitor were added and the reaction was incubated at 37 ℃ for 2 min; (3) 1 μL of MMLV reverse transcriptase was added and

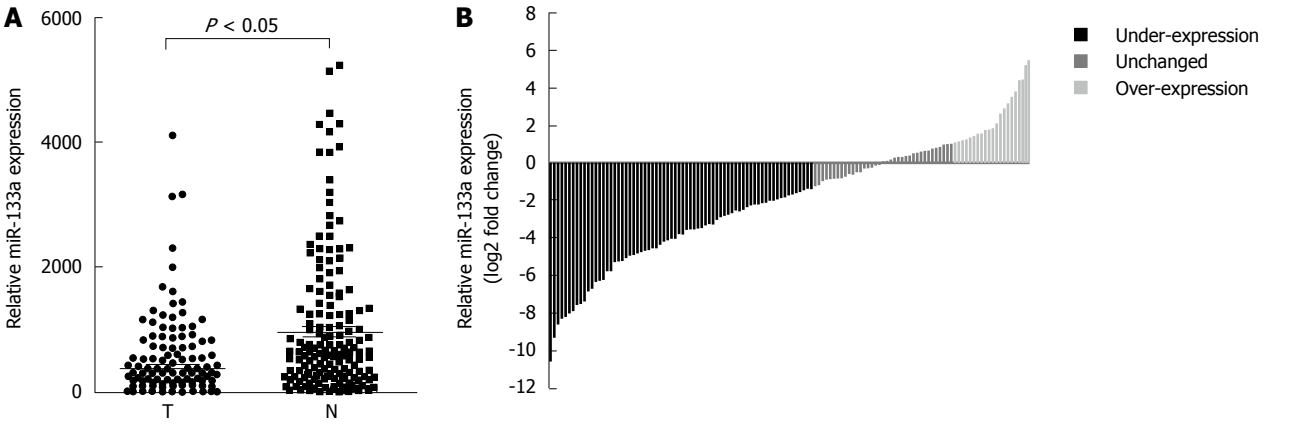

Figure 1 MiR-133a expression is downregulated in colorectal cancer patients. A: MiR-133a expression was determined by quantitative real-time polymerase chain reaction and normalized against U6 RNA (an endogenous control). Expression was compared between 169 pairs of colorectal cancer tissues (T) and adjacent non-tumorous tissues (N); B: Fold changes of miR-133a of individually matched samples. Data are represented as $\log_2$ fold change (T/N) (< -1, under-expression; -1, unchanged; > 1, over-expression).

Table 1 Demographic characteristics of patients with colorectal cancer

Characteristic	Number of cases (n = 175)	miR-133a expression median (IQR)	P value
Sex			0.727
Male	96	127.40 (34.93-535.51)	
Female	73	135.30 (31.90-435.10)	
Age (yr)			0.800
≤ Median	85	173.72 (39.43-603.81)	
> Median	84	109.90 (27.18-311.64)	
Tumor location			0.484
Colon	79	135.30 (27.31-468.70)	
Rectum	90	132.35 (43.73-525.97)	
Differentiation			0.004
Well	18	560.88 (98.18-1027.33)	
Moderate	130	134.45 (35.88-434.56)	
Poor	21	35.33 (26.05-145.77)	
Size			0.527
≤ 5 cm	101	129.59 (31.90-468.70)	
> 5 cm	68	135.35 (35.87-524.93)	
Local invasion			< 0.001
T1-T2	42	562.75 (299.65-999.73)	
T3-T4	127	88.17 (25.50-267.13)	
Lymph node metastasis			< 0.001
No	98	300.87 (72.88-740.23)	
Yes	71	67.92 (21.22-200.36)	
TNM stage			< 0.001
Stage Ⅰ	34	708.05 (362.41-1054.28)	
Stage Ⅱ	64	132.50 (34.53-421.89)	
Stage Ⅲ	71	67.92 (21.22-200.36)	

TNM: Tumor node metastasis; IQR: Interquartile range.

the final reaction was incubated at 37 ℃ for 50 min, followed by 70 ℃ for 5 min. For quantitative real-time polymerase chain reaction (qRT-PCR), the amplification protocol was carried out in the ABI PRISM 7500 Sequence Detection System (Applied Biosystems Inc., Waltham, MA, United States) as follows: initiation at 95 ℃ for 1 min, followed by 45 cycles of 95 ℃ for 15 s, 60 ℃ for 30 s, 72 ℃ for 45 s, followed by a dissociation protocol. The U6 small nuclear RNA was used as a reference gene to normalize RNA concentrations, and the relative expression of miR-133a in Caco-2 cells was used as a calibrator by the comparative threshold cycle (Ct) method ($2^{-\Delta\Delta Ct}$). Triplicate quantification tests were performed and the average was calculated for each sample.

Statistical analysis

All statistical analyses were performed using SPSS 17.0 statistical software (IBM Corporation, Chicago, IL, United States). The median concentrations of miR-133a were compared among different groups using a Mann-Whitney U test or Kruskal-Wallis test. OS curves were calculated using the Kaplan-Meier method and the statistical differences between subgroups were compared by the log-rank test. The Cox proportional hazards regression model was employed for univariate and multivariate analyses to estimate the prognostic factors for survival prediction. Data are expressed as median and interquartile range. $P < 0.05$ was considered statistically significant.

RESULTS

Decreased expression of miR-133a in CRC

To reveal the role of miR-133a in CRC, qRT-PCR was performed to measure miR-133a levels in 169 pairs of CRC tissues and adjacent non-cancerous tissues. Median miR-133a levels were significantly lower in CRC tissues compared with matched non-cancerous tissues [133.6 (33.73-508.2) *vs* 804.8 (298.64-1727.5), $P < 0.05$] (Figure 1A). In addition, the miR-133a expression was found to be decreased at least 2-fold compared with adjacent non-cancerous tissue in 55.6% (94/169) of cases (Figure 1B).

Correlations between miR-133a expression and CRC clinicopathologic characteristics

The relationship between miR-133a expression and clinicopathologic parameters was evaluated. As shown in Table 1, the level of miR-133a in CRC was strongly correlated with tumor differentiation, local invasion, lymph

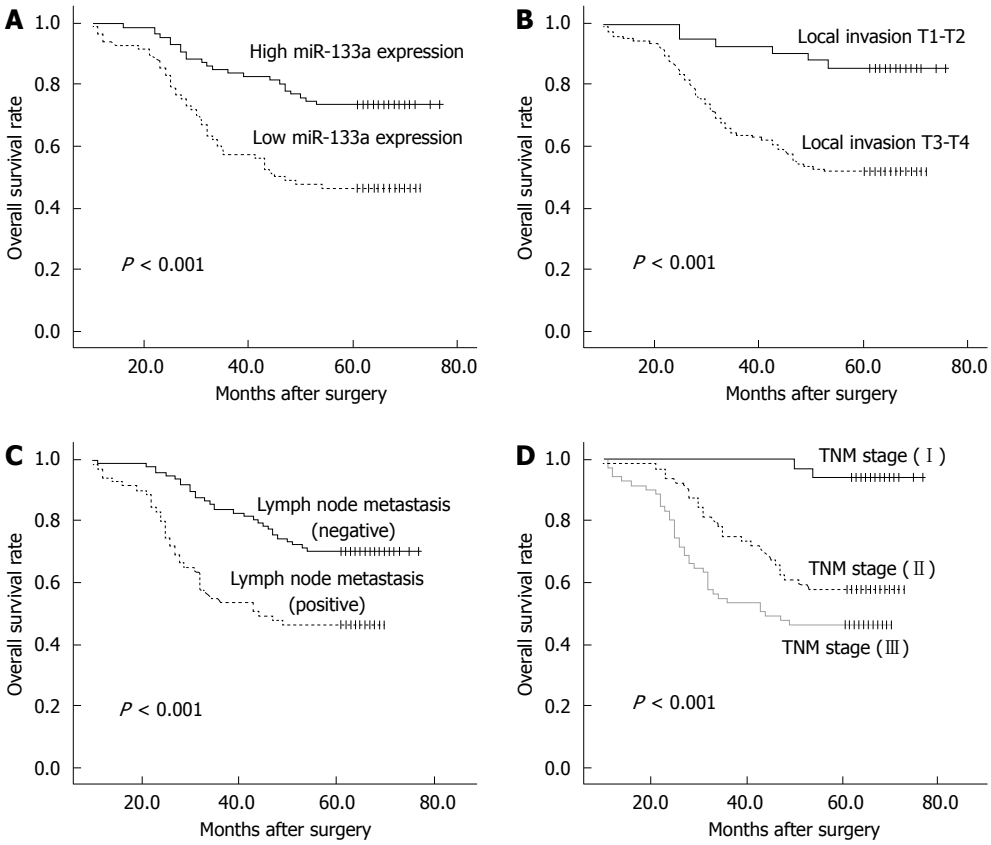

Figure 2 Kaplan-Meier curves for overall-survival in 169 colorectal cancer patients. A: MiR-133a level; B: Local invasion; C: Lymph node metastasis; D: Tumor-node-metastasis stage (TNM).

node metastasis and clinical TNM stage ($P < 0.05$ for all). However, there were no significant associations between miR-133a expression and other clinical features including sex, age, tumor location and tumor size. Taken together, these observations indicate that miR-133a expression is downregulated in CRC and is associated with the disease progression.

Correlation between miR-133a expression and OS in CRC patients

Of the 169 CRC patients, 67 died within the follow-up period, resulting in a cumulative 5-year OS rate of 60.4%. The prognostic value of miR-133a expression in CRC OS was evaluated between patients with low and high miR-133a expression levels. Patients with miR-133a expression levels below the median level (133.6) were assigned to the low expression group (median 33.73, $n = 85$), and those with values above the median were assigned to the high expression group (median 514.9, $n = 84$). Kaplan-Meier analysis showed that the cumulative 5-year OS rate was 46.3% in the low expression group, and 73.6% in the high expression group (Figure 2A). The log-rank test showed that the OS rate of patients with low miR-133a expression was significantly poorer than that of the remaining cases ($P < 0.001$). OS was also significantly associated with local invasion (Figure 2B; $P < 0.001$), regional lymph node metastasis (Figure 2C; $P < 0.001$) and TNM stage (Figure 2D; $P < 0.001$).

Independent prognostic indicators for CRC patients

A Cox proportional hazards regression model analysis was performed to determine the independent prognostic indicators for patients with CRC. The results of univariate analyses revealed statistically significant correlations between OS and miR-133a level (HR = 0.385, 95%CI: 0.232-0.638, $P < 0.001$), tumor local invasion (HR = 4.328, 95%CI: 1.780-10.020, $P < 0.05$), lymph node metastasis (HR = 2.416, 95%CI: 1.488-3.923, $P < 0.001$) and TNM stage (HR = 2.336, 95%CI: 1.609-3.393, $P < 0.001$) (Table 2). A multivariate analysis of these factors showed that miR-133a level and TNM stage maintained their significance as independent prognostic factors for OS (HR = 0.590, 95%CI: 0.350-0.995, $P < 0.05$; and HR = 6.111, 95%CI: 1.029-36.278, $P < 0.05$, respectively).

DISCUSSION

There is mounting evidence demonstrating the tissue-specificity and stability of miRNA expression patterns in various tumors, which has provided insights into the molecular mechanisms involved[14]. Although the precise mechanisms are unknown, many functional studies suggest that miRNA dysregulation is involved in the initiation and progression of cancer[15-17]. miR-21 and miR-92a, two highly investigated miRNAs that function as promoters for cell proliferation, invasion and metastasis, are significantly overexpressed in CRC[18]. Recently, the

Table 2 Univariate and multivariate analyses for overall survival in colorectal cancer patients

Variable	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Age (yr)	1.107	0.685-1.789	0.679			
Sex (male vs female)	1.154	0.714-1.863	0.559			
Tumor location (rectum *vs* colon)	1.006	0.623-1.625	0.981			
Tumor size	1.318	0.814-2.134	0.261			
Differentiation	0.788	0.490-1.268	0.326			
Local invasion (T3-4 *vs* T1-2)	4.328	1.780-10.020	0.001	1.196	0.424-3.370	0.735
Lymph node metastasis	2.416	1.488-3.923	< 0.001	0.238	0.033-1.717	0.154
TNM stage	2.336	1.609-3.393	< 0.001	6.111	1.029-36.278	< 0.05
miR-133a expression (high *vs* low)	0.385	0.232-0.638	< 0.001	0.590	0.350-0.995	< 0.05

TNM: Tumor node metastasis; HR: Hazard ratio.

downregulation of other miRNAs, such as miR-126 and miR-218, has also been implicated in CRC[19,20]. Contradictory data concerning the role of miR-133a in the development and progression of CRC have emerged, with evidence that it can behave both as a promoter and a suppressor. To reconcile this discrepancy, the expression patterns of miR-133a in CRC tissues were examined, along with their association to CRC development.

The expression of miR-133 has been characterized in multiple species and found to play roles in the development of different types of malignant tumors. Results of this study indicated that miR-133a is significantly downregulated in CRC tissues compared with the adjacent normal tissues, which is consistent with previous studies showing a reduction in miR-133a in CRC patients compared to healthy controls. Furthermore, the levels of miR-133a were significantly lower in tumors with poor differentiation, greater depth of local invasion, positive lymph node metastasis and advanced disease. Hamara *et al*[21] reported that miR-133a is homologous to the 3'-UTRs of iron-related genes and consistently reduced in CRC patients in comparison to healthy colon mucosa. These findings suggest that the downregulation of miR-133a is related to the tumorigenesis and progression of CRC. The precise mechanism for this regulation is unclear, however, there is evidence to suggest that it involves the inhibition of the MAPK pathway[12].

To explore the potential prognostic value of miR-133a, the relationship between expression levels and OS in CRC patients was analyzed. Results indicate that patients with low expression had poorer survival compared to those with high expression of miR-133a. Furthermore, miR-133a and TNM stage could serve as independent prognostic indicators for the survival of CRC patients, indicating that the detection of miR-133a together with pathologic diagnosis in tumor tissues could be used to evaluate patient outcome and help design optimal individual treatment strategies.

A previous study reported that miR-133a in CRC patients may serve as a gene promoter for brain metastasis[13]. However, six of the CRC patients in the present study with liver metastasis had lower expression of miR-133a, and were not analyzed for the prognostic factor due to the limited number of cases. This discrepancy may indicate distinct organ-specific miR-133a functions, induced by different target genes. For example, miR-133a induces G2 arrest in renal cell carcinomas[7], and inhibits breast cancer cell growth and invasion by regulating fascin 1 expression, and thus its downregulation is associated with poor survival in breast cancer patients[6]. In esophageal squamous cell carcinoma, miR-133a was reported to regulate the expression of CD47 and function in the development of this cancer[22]. Other validated oncogenic targets of miR-133a include fascin homolog 1, glutathione-*S*-transferase pi 1 and transgelin 2 in bladder cancer[23-25], and actin-related protein 2/3 complex, subunit 5 in lung squamous cell carcinoma[5].

In conclusion, the results of this study confirm the clinical and prognostic significance of miR-133a in CRC. Although this study was limited by the retrospective design and the relatively small number of CRC patients, the data suggest that miR-133a plays a critical role in the development and progression of CRC and therefore may serve as a potential therapeutic target. Future studies evaluating the role of miR-133a in the metastasis of various cancers will help to further elucidate the mechanisms controlling miR-133a dysregulation and define the oncogenic targets.

COMMENTS

Background

Colorectal cancer (CRC) is one of the most common malignancies worldwide, with over 1.2 million new cases and an estimated 608700 deaths occurring in 2008 alone. Although recent advances have been achieved in comprehensive therapeutic strategies, CRC outcome remains poor. Biomarkers such as carcinoembryonic antigen have been recommended for CRC prognosis and postoperative surveillance, despite limited sensitivity. Therefore, the identification of novel and reliable biomarkers is urgently needed for CRC prognosis.

Research frontiers

There is increasing evidence for microRNA (miRNA) dysregulation in tumor development, which can be utilized as valuable biomarkers. Previous data have indicated that the ectopic expression of miR-133a is associated with various human malignancies. However, one study showed that miR-133a repressed tumor growth and metastasis, while another study indicated that miR-133a serves as a gene promoter for brain metastasis. The present study confirms the repression of miR-133a in CRC tissues, and provides evidence that miR-133a expression can serve as a reliable prognostic indicator for the progression of CRC.

Innovations and breakthroughs

In the present study, miR-133a levels were measured in CRC tissues by quantitative real-time PCR, and the clinical significance of miR-133a expression was investigated. The data indicate that the expression of miR-133a was reduced in CRC tissues and was significantly associated with tumor differentiation, local invasion, regional lymph node metastasis and TNM stage. Moreover, CRC patients with low miR-133a expression had poorer overall survival than those with high miR-133a expression. Multivariate analysis suggested that miR-133a is an independent factor for CRC prognosis.

Applications

The confirmation of miR-133a dysregulation in CRC and its correlation with disease progression suggest that miR-133a is a potential novel target for therapeutic strategies. Moreover, results of this study may help to develop the use of miR-133a levels as a biomarker to independently predict clinical outcome.

Terminology

MicroRNAs are a class of short non-coding ribonucleotides that have been shown to regulate gene expression. Multiple miRNAs have been implicated in the initiation and progression of tumors through dysregulation of oncogenes and tumor suppressor genes, including miR-133a, which may be involved in the development of different types of malignant tumors. The three known genes of miR-133 include miR-133a-1, miR-133a-2 and miR-133b, located on chromosomes 18, 20 and 6, respectively.

Peer review

The authors present a study concerning the clinical implication of miR-133a expression in colorectal cancer. The results demonstrate that miR-133a is reduced in CRC tissues, and low expression is associated with poorer overall survival of CRC patients. These data indicate that miR-133a levels may serve as a useful prognostic biomarker for clinical assessment of patient outcome, and implicate miR-133a as a potential therapeutic target for CRC.

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P- Reviewer: Haier J, Il Kim T, Seow-Choen F **S- Editor**: Ma YJ
L- Editor: Wang TQ **E- Editor**: Wang CH

World Journal of Gastroenterology

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DOI: 10.3748/wjg.v20.i32.11347

World J Gastroenterol 2014 August 28; 20(32): 11347-11355
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

RETROSPECTIVE STUDY

MicroRNA-218 is upregulated in gastric cancer after cytoreductive surgery and hyperthermic intraperitoneal chemotherapy and increases chemosensitivity to cisplatin

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Supported by The PhD Start-up Funds of Guangzhou Medical College, Guangdong Province, China, No. 2012C66 and No. 2012C69; Guangdong Province Natural Science Fund, No. S2013010016662; and the National Natural Science Foundation of China, No. 81201932 and No. 81372493
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Received: January 5, 2014 Revised: April 9, 2014
Accepted: May 19, 2014
Published online: August 28, 2014

Abstract

AIM: To investigate the molecular mechanisms of miRNA in advanced gastric cancers (AGCs) before and after cytoreductive surgery (CRS) + hyperthermic intraperitoneal chemotherapy (HIPEC).

METHODS: A miRNA microarray containing human mature and precursor miRNA sequences was used to compare expression profiles in serum samples of 5 patients with AGC before and after CRS + HIPEC. The upregulation of miR-218 was confirmed by real-time reverse transcription polymerase chain reaction and its expression was analyzed in SGC7901 gastric cancer cells.

RESULTS: miRNA microarray chip analysis found that the level of miR-218 expression was upregulated more than 8 fold after CRS + HIPEC. Furthermore, miR-218 increased gastric cancer cell chemosensitivity to cisplatin *in vitro* and inhibited gastric cell tumor growth in nude mice *in vivo* (0.5 *vs* 0.78, $P < 0.05$).

CONCLUSION: Our results indicated that targeting miR-218 may provide a strategy for blocking the development of gastric cancer and reverse the multi-drug resistance of gastric cell lines.

Key words: Advanced gastric cancer; Cytoreductive surgery; Hyperthermic intraperitoneal chemotherapy; miR-218; Multi-drug resistance; MicroRNA

Core tip: MicroRNAs (miRNAs) are short single-stranded RNAs associated with gene regulation at the transcriptional and translational levels. miRNA up- or down regulation has been linked to cancer development. We analyzed miRNA expression in advanced gastric cancers before and after cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC) to gain further understanding of the molecular mechanisms of CRS + HIPEC. Furthermore, we described the regulation and function of miR-218 in gastric cancer emphatically. Our results indicated that targeting miR-218 may provide a strategy for blocking the development of gastric cancer and can reverse the multi-drug resistance of gastric cell lines.

Zhang XL, Shi HJ, Wang JP, Tang HS, Wu YB, Fang ZY, Cui SZ, Wang LT. MicroRNA-218 is upregulated in gastric cancer after cytoreductive surgery and hyperthermic intraperitoneal chemotherapy and increases chemosensitivity to cisplatin. *World J Gastroenterol* 2014; 20(32): 11347-11355 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11347.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11347

INTRODUCTION

Gastric cancer is the second leading cause of cancer death in the world, with often a poor prognosis due to the advanced stage of the disease at diagnosis. The peritoneum is the most common site of metastasis in patients with gastric cancer[1]. Although surgery and chemotherapy can be curative for early-stage disease, the prognosis for patients with advanced disease is poor. In China, advanced gastric cancer (AGC) is the third leading cause of cancer deaths, with 300000 deaths per year[2]. In addition to hematogenous and lymphatic metastasis, patients with AGC may have free cancer cells dissemination in the peritoneal cavity, leading to peritoneal carcinomatosis (PC). PC may be present in 5%-20% of patients undergoing gastrectomy of AGC. The prognosis and survival of patients with gastric originated PC are extremely poor[3,4].

2010 French guidelines designated cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC) as the standard method for treating colorectal PCs[5], and evaluated the effectiveness of CRS + HIPEC treatment for other primary tumors, including gastric cancer, ovarian, and sarcoma[6-8]. CRS + HIPEC can reduce visible tumor burden and HIPEC can eradicate micro-metastases[9-12], improving outcomes in patients with AGC of PC[13].

HIPEC was performed by our self-developed "BR-TRG Ⅱ type high-precision hyperthermic perfusion intraperitoneal treatment system" with precise ± 0.2 ℃ temperature control, ± 5% flow control accuracy, and an automatic cooling function.

Patients completed the first session of HIPEC in the operating room under general anesthesia. At the first session, 5-fluorine and cisplatin were added into the 0.9% sodium chloride (3500-5000 mL) as perfusion liquid. HIPEC was then performed for 60 min with a velocity of 450-600 mL/min and an inflow temperature of 43 ± 0.2 ℃.

Several non-randomized comparative studies have suggested that CRS + HIPEC is superior to CRS alone in patients with AGC[14,15]. Although CRS + HIPEC is an accepted method of treatment, its exact molecular mechanisms have not been fully elucidated.

MicroRNAs (miRNAs) are small non-coding RNA molecules with gene regulation capabilities[16,17]. Mature miRNAs bind to 3' untranslated regions of target mRNA, leading to the silencing of mRNA. These miRNAs have been found to regulate complicated biological behaviors, including cell proliferation, differentiation, and death. Specific miRNAs have been found to be associated with various types of cancer, including miR-34a, miR-21, miR-16, and miR-92a in breast cancer[18-20]; miR-101 in gastric cancer[21,22]; miR-130a, miR-203, miR-205, and miR-21 in prostate cancer[23,24]; miR-182 in melanoma[25]; and miR-92b and miR-9/9* in brain tumors[26]. In contrast, few miRNAs have been shown to be associated with AGCs after CRS and HIPEC. The importance of miRNAs in cancer was highlighted by the discovery that more than 50% of miRNA target cancer-associated genomic regions or fragile sites, including lung, breast, brain, liver, colon cancer, and leukemia[27,28].

Since the molecular mechanisms of miRNA expression in AGCs treated with CRS + HIPEC remain elusive, further research is needed to examine the contribution of miRNAs to the mechanisms of carcinogenesis and their association with patient prognosis. We therefore utilized miRNA microarray chips to analyze the expression profiles of miRNAs in five patients with AGC being treated with CRS + HIPEC. We found that miR-218 was upregulated by more than 8 fold, which was confirmed by polymerase chain reaction (RT-PCR). These findings indicate the molecular basis of CRS + HIPEC effects in patients with AGC.

To further define the function of miR-218, it was upregulated in human gastric cancer cells (SGC7901), and cell vitality and chemosensitivity were then studied.

MATERIALS AND METHODS

Patients

Five patients with gastric PC, 4 men, and 1 woman, aged 46-75 years (median, 50.3 ± 1.6 years) were recruited from January to June 2012 (Table 1) and treated with CRS + HIPEC. Eligibility criteria included the following: (1) no prior chemotherapy or chemotherapy administered more than 6 mo ago; (2) an Eastern Cooperative Oncology Group performance score of 0-2; (3) adequate bone marrow, hepatic, and renal functions; and (4) 18-75 years of age. All diagnoses of AGC were confirmed histologically. Pre- and post-operative serum samples were collected from the 5 patients. All patients provided written informed consent, and the study protocol was approved by the ethics committee of Guangzhou Medical College and was in accordance with the Helsinki Declaration on ethical principles for medical research involving human subjects.

CRS and HIPEC

All CRS and HIPEC procedures were performed by the same team of surgical oncologists and anesthesiologists. Abdominal exploration was performed according to previously reported methods[29]. The volume and character of ascites were also recorded. After evaluation, patients were scheduled for CRS and HIPEC[30]. HIPEC used to manage peritoneal surface malignancies was in accordance with the standard protocol of our center. Hy-

Table 1 Clinical background of 5 patients with advanced gastric cancer

Age	Sex	TNM stage	Histology of tumor	Drug regimen of HIPEC	Duration of HIPEC (min)	ECOG	HIPEC temperature (℃)	Time of surgery (h)	Following time (yr) and results
49	Male	T4aN1M0	Poorly-differentiated adenocarcinoma	5-Fu cisplatin	60	1	43	3	1, alive
56	Male	T3N2M0	Moderately-differentiated adenocarcinoma	5-Fu cisplatin	60	2	43	3.5	1.5, alive
67	Female	T4N2M0	Poorly-differentiated adenocarcinoma	5-Fu cisplatin	60	0	43	3.8	1, dead
53	Male	T3N1M0	Poorly-differentiated adenocarcinoma	5-Fu cisplatin	60	1	43	4	1.2, dead
62	Male	T4bN0M0	Moderately-differentiated adenocarcinoma	5-Fu cisplatin	60	2	43	3	0.5, alive

ECOG: Eastern Cooperative Oncology Group; HIPEC: Hyperthermic intraperitoneal chemotherapy.

perthermic perfusion was performed with 4 L of heated saline supplied with 60 mg of cisplatin and 1000 mg of 5-FU. Human serum samples were collected from the 5 AGC patients 1 h before and 1 h after CRS + HIPEC, which were quickly frozen in liquid nitrogen and stored at -80 ℃.

RNA isolation

Total RNA was isolated from samples using TRIzol kits (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's protocol. Yield and quality of RNA were assessed by ultraviolet absorbance at 260 nm and 280 nm.

miRNA microarrays

miRNA was isolated from the total RNA sample using mirVana miRNA Isolation Kits (Ambion Inc., Austin, TX, United States). The miRNA quantity was assessed by a fluorometer (Bio-Rad, Hercules, CA, United States) and by 1% formaldehyde agarose gel electrophoresis. Expression of miRNAs was assayed using microRNA chips (Exiqon, Vedbaek, Denmark). Serum samples were sent to KangChen-Biotech (Shanghai, China) for array hybridization, background subtraction, and normalization. Total RNA (5 μg) was labeled with Cy3 modified RNA linker at the 3'-end using T4 RNA ligase. Briefly, the reaction was carried out in 3 μL volumes at 37 ℃ for 30 min containing 2.0 μL RNA, 1.0 μL of CIP buffer, and CIP (Exiqon) mixture. The reaction was terminated by heating at 80 ℃ for 3 min. Images were analyzed with Genepix Pro 6.0 (Axon). Data were then collected from three independent experiments and miRNA with at least 2-fold changes were selected for further analysis.

Two step real-time RT-PCR

Real-time quantitative PCR was performed using SYBR Green PCR kits (SYBR biopars, GUASNR, Iran). The reaction was carried out in a 20 μL volume mixture, containing 300 nM of each primer (forward 5'-AAGACACCCTGGACGAAGCC-3', reverse 5'-ACAACCAGAGTCCACCGGCG-3'), 10 ng of cDNA.

DNA was amplified with an initial denaturation at 94 ℃ for 3 min, followed by 35 cycles of 94 ℃ for 15 s and 59.8 ℃ for 15 s (gain set at 10 for SYBR Green). Each experiment was carried out in triplicate. The amplification efficiency was assessed based on the slope of a linear regression model. Ten-fold dilutions of each cDNA were used as a PCR template.

Cell culture

A human gastric cancer cell line SGC7901 was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin at 37 ℃ in 5% CO_2.

Generation of SGC7901 cells with stable expression of miR-218

SGC7901 cells were first transfection with pCR-miR-218 or pCR-miR-NC, and then selected in 1 mg/mL G418 (Invitrogen) supplied complete DMEM. miR-218 or miR-NC stable overexpressed cells were obtained after 3-4 wk of selection. The chemosensitivity of these cells were evaluated using a WST-1 kit (Roche) according to the manufacturer's instruction at the indicated times. Each experiment was carried out in triplicate.

Anchorage-independent colony formation assay

The soft agar colony formation assay was used to monitor anchorage-independent growth. Briefly, agar plates were prepared using 1% agarose (Sigma, Saint Louis, MI, United States). Plates were stored at 4 ℃ and pre-warmed in 37 ℃ before use. Top agar (0.5%) containing 10^3 cells were then added onto the well. After 3-4 wk of incubation, colonies were assessed after methanol fixation and 0.1% crystal violet staining. Colonies with a diameter greater than 2 mm were counted. Each experiment was carried out in triplicate.

In vitro assay of chemosensitivity to cisplatin

6×10^3 cells were first seeded in each well of 96-well plates. After overnight incubation, cisplatin was added into each well, resulting in the final concentration of 0 to 256 μmol/L. Cell viability was determined using WST-1 kit after 72 h. IC_{50} was obtained from three independent

Table 2 Three-fold upregulation of microRNAs in advanced gastric cancer tumor serums

MicroRNA	Raising multiples	*P* value
miR-218	8.419	0.004
miR-135b	5.317	0.003
miR-135a	4.788	0.016
miR-433	4.401	0.025
miR-409	4.389	0.039
miR-96	4.280	0.050
miR-647	4.113	0.067
miR-376a	3.940	0.003
miR-4326	3.899	0.045
miR-1306	3.705	0.021
miR-937	3.699	0.028
miR-326	3.661	0.007
miR-214	3.500	0.001
miR-377	3.403	0.009
miR-661	3.397	0.005
miR-3678	3.235	0.011
miR-632	3.226	0.035
miR-154	3.140	0.028
miR-134	3.119	0.027

experiments. Cisplatin at twice the concentration of IC_{50} were used in the following studies.

In vivo tumorigenesis study

Twenty four BALB/c nude mice (female, 6-9 wk, Guangzhou Laboratory Animal Center, Chinese Academy of Sciences, Guangzhou, China) were used in the study. Mice were maintained in a special pathogen-free (SPF) house with 12 h alternating light and dark cycles, and were given adequate nutrition and water *ad libitum*. 6×10^6 of cells suspended in FBS-free DMEM were injected into each side of the posterior flank of nude mice subcutaneously. Mice were sacrificed and tumors were collected 30 days after implantation. Tumor volume was calculated as follows: volume = 0.5 × length × width^2. Experimental protocols were reviewed and approved by the Animal Ethics Committee of Guangzhou Medical College.

Statistical analysis

Quantitative data were presented as mean ± SD. Comparisons between the quantitative data were made using the *t* test. SPSS 16.0 was used in the statistical analysis and $P < 0.05$ was considered statistically significant.

RESULTS

miRNA expression profiles in sera of patients with AGC before and after CRS + HIPEC

miRNA expression profiles of paired serum samples of 5 AGC patients before and after CRS + HIPEC were analyzed by miRCURY™ bead-based flow LNA microarray platform, using 5S RNA for normalization. The miRNA expression patterns differed significantly (Figure 1). Of the miRNAs assayed, miR-218 was upregulated by more than 8-fold (Table 2).

Real-time PCR validation of microarray results

Results of the microarray were further validated by quantitative RT-PCR, including the results of miR-218, miR-135a, miR-409, and miR-96. The RT-PCR results were consistent with the microarray data (Figure 2).

Upregulation of miR-218 suppressed in the proliferation of gastric cancer

MiR-218 was reported to be downregulated in gastric cancers[31] and was upregulated after CRS + HIPEC. To explore the roles of miR-218 in drug resistance, SGC7901 gastric cell line with a stable overexpression of miR-218 were established by transfecting miR-218 plasmids and then undergoing cisplatin-based selection. Meanwhile, the SGC7901/miR-NC cell line was established as a control. The overexpression of miR-218 in SGC7901/miR-218 cells was confirmed by RT-PCR (Figure 3C). Cell growth was measured using WST-1 kit at indicated time points.

The results showed that upregulation of miR-218 markedly inhibited the proliferation of SGC7901 cells (Figure 3A). The anchorage independent colony formation assay also demonstrated the same results (Figure 3B).

MiR-218 increased chemosensitivity of gastric cancer cells to cisplatin via its target mTOR inhibitor

To investigate whether miR-218 plays an important role in the drug resistance of gastric cancer, gastric tumor cell lines, SGC7901/miR-NC, SGC7901/miR-218, or SGC7901/miR-218 using mTOR inhibitor rapamycin and different concentrations of cisplatin (0 to 256 μmol/L) for 72 h (Figure 4A). Cell viability was measured using the WST-1 kit. The results showed that overexpression of miR-218 increased sensitivity of SGC7901 cells to cisplatin, while rapamycin can further enhance chemosensitivity. Cisplatin at 10 μmol/L showed a remarkable inhibition of cell proliferation (Figure 4B).

MiR-218 impaired in vivo tumor growth

The chemosensitization role of miR-218 was further explored using ectopic transplantation in a nude mice model. Stable cell lines, SGC7901/miR-218, and SGC7901/miR-NC were injected into nude mice subcutaneously. Tumor growth in the miR-218 group was reduced significantly when compared to the control group (Figure 5A and B). The tumor weights of the xenograft in the miR-218 group were greater than those in the miR-NC group (Figure 5C). These data indicated the suppressor role of miR-218 in gastric cancer.

DISCUSSION

PC of gastrointestinal or primary origin is considered invariably fatal, with only palliative treatment possible. To date, no standard treatment has been developed for PC from AGC, although the original trial of CRS + HIPEC resulted in a mean overall survival of 7.2 ± 4.6 mo with acceptable morbidity[32]. This new treatment modality has

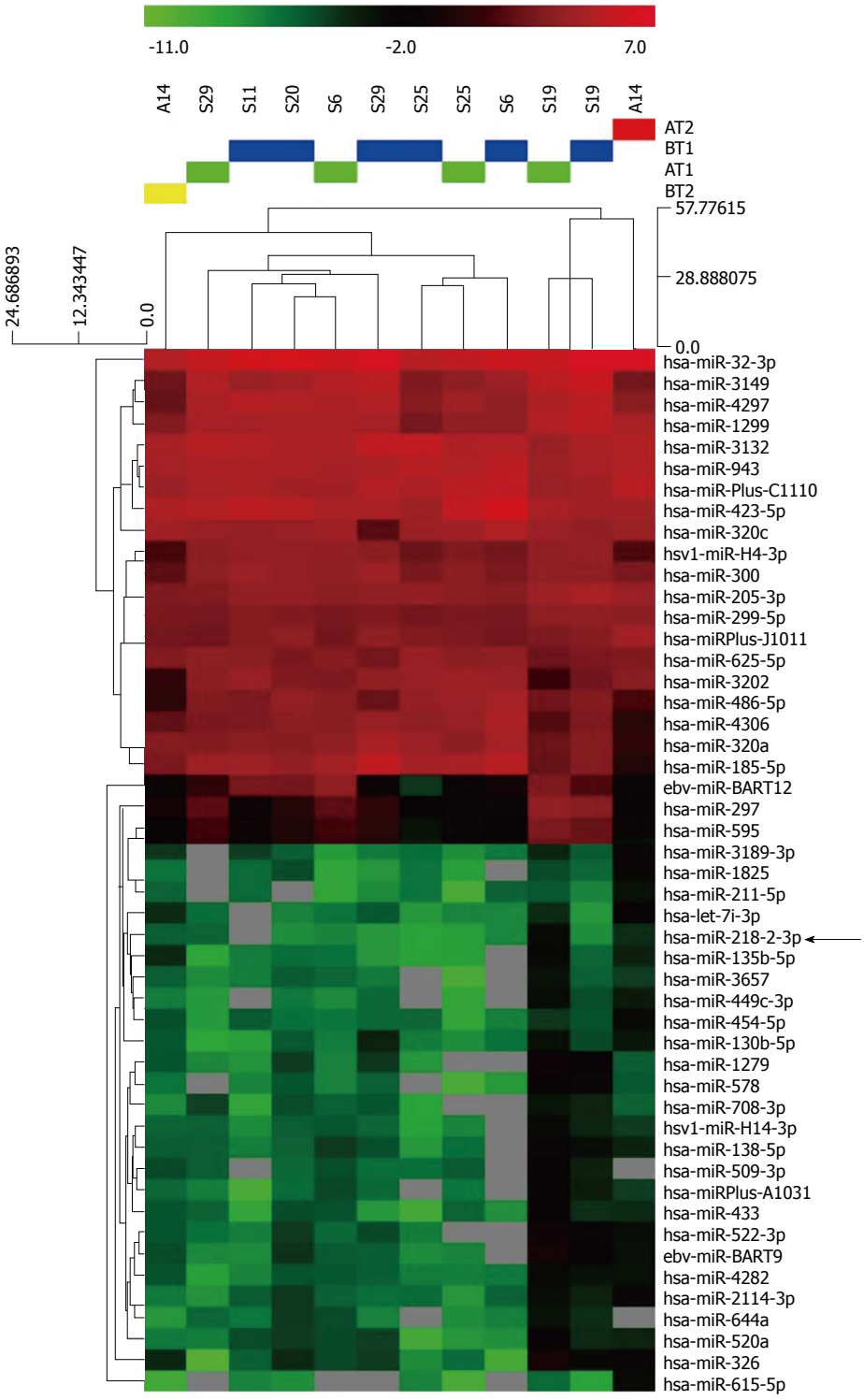

Figure 1 Hierarchical clustering of microRNA in advanced gastric cancer serum samples. Advanced gastric cancer (AGC) serum samples were clustered according to the expression profile of 86 differentially-expressed microRNAs (miRNAs) of the paired serum samples of 5 AGC patients collected 1 h before and 1 h after cytoreductive surgery plus hyperthermic intraperitoneal chemotherapy. Samples were in columns and miRNAs in rows. The *P* values for these miRNAs were less than 0.05 in AGC serum samples.

gradually gained acceptance in many countries. A meta-analysis of 13 randomized controlled trials found that HIPEC showed marked improvements of survival in patients with AGC when compared with current treatments[33]. As a result, CRS + HIPEC was recommended as the optimal treatment for AGC by a panel of international experts[34]. Nevertheless, this treatment modality remains controversial, and additional high-quality clinical studies are still needed in order to prove its effectiveness.

Changes in miRNA expression have been found to contribute to the initiation and progression of cancer. The relationship between miRNAs and tumors has

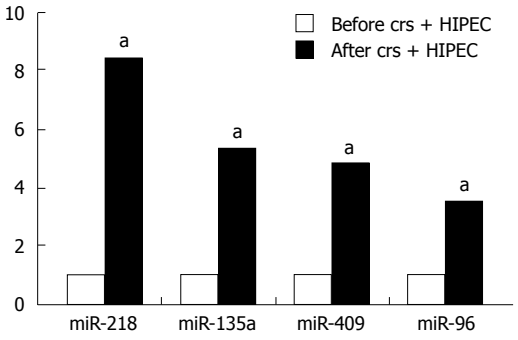

Figure 2 Upregulation of microRNA after cytoreductive surgery and hyperthermic intraperitoneal chemotherapy treatment. $^{a}P < 0.05$ *vs* control.

suggested that miRNAs may be altered by treatment in patients with AGC. We therefore analyzed paired serum samples from patients with AGC that were obtained before and after CRS + HIPEC. We found that miR-218 was upregulated by more than 8 fold after CRS + HIPEC. Further analysis using computer-aided algorithms (TargetScan and PicTar software) predicted that more than 30 genes were potential targets of miR-218, some of which are associated with multi-drug resistance (MDR). Thus, together with previous studies[35-36], our bioinformatics results suggest that miR-218 may be altered by HIPEC.

In the present study, we focused on the effect of miR-218 on AGC metastasis and found that miR-218 was a tumor suppressor in AGC metastasis. Furthermore, miR-218 increased chemosensitivity to cisplatin *in vitro* and *in vivo* by inducing apoptosis. Cisplatin is currently used in the treatment of many types of advanced cancer, including gastric cancer, due to the broadest-spectrum anticancer capability[37]. Currently, the most effective systemic treatment for metastatic gastric cancer consists of cisplatin-based combination chemotherapy[38]. However, chemoresistance is still the most major and frequent obstacle to effective treatment[39]. Our data show that miR-218 overexpression can reverse the MDR of cisplatin and can increase the chemosensitivity of gastric cancer cells. When compared to the mTOR inhibitor rapamycin, the effect is better than miR-218 alone. Therefore, miR-218 may be used as a biomarker to confirm the extensiveness of tumor resection and to evaluate the efficacy of HIPEC.

The key finding in our study is that miR-218 suppressed the proliferation of tumor cells and inhibited the mTOR signaling pathway in gastric cancer. In addition, upregulation of miR-218 increased chemosensitivity of gastric cancer cells to cisplatin via its target mTOR.

In conclusion, we have identified several miRNAs whose expression was affected before and after CRS + HIPEC in AGC. The upregulation of miR-218 can inhibit AGC cell invasion and metastasis, as well as further reverse the MDR of cisplatin. The results indicate that restoration of miR-218 may be a rational therapeutic strategy for the treatment of AGC in the future. Impor-

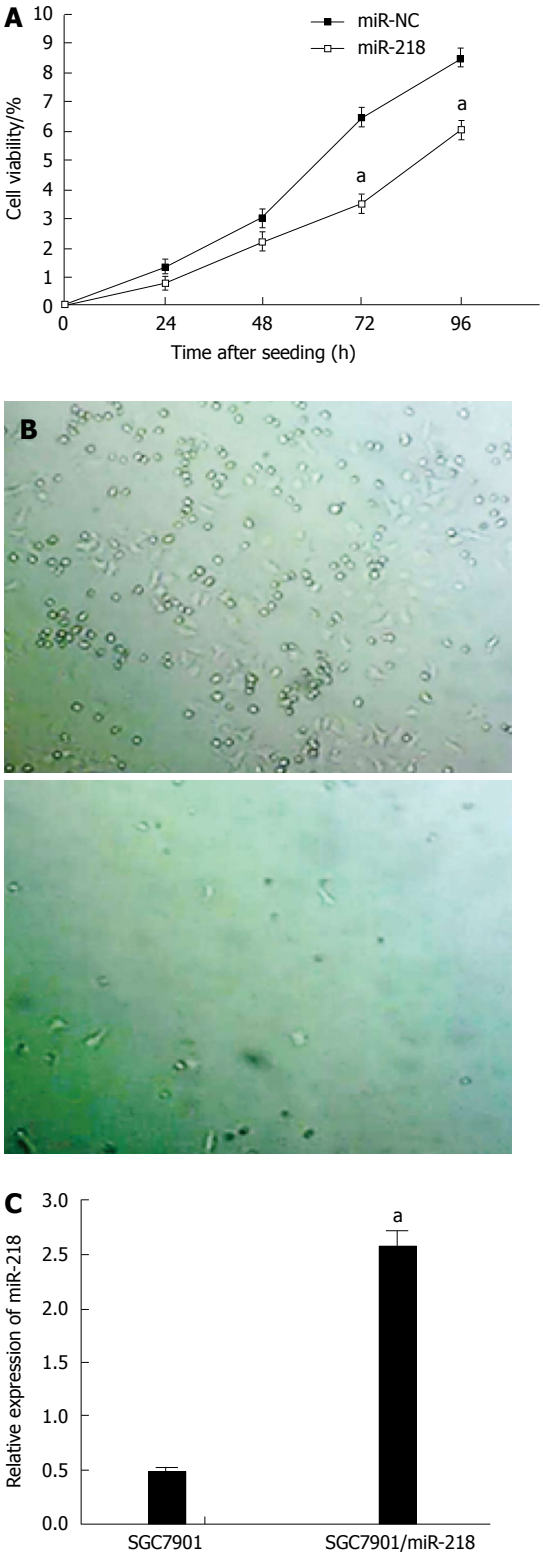

Figure 3 Upregulation of miR-218 inhibited growth of gastric cancers. A: The upregulation of miR-218 inhibited SGC7901 (human gastric cancer cells) cell growth. Cell viability was measured using a WST-1 kit at indicated time points. Data are presented as mean ± SD from three independent experiments performed in sextuple; B: miR-218 overexpression reduced colony formation of SGC7901 cells. 103 cells were mixed with agarose and seeded in 9-well plates for two weeks; C: The expression levels of miR-218 in the parental SGC7901 and the SGC7901/miR-218 stable cell line. Data are presented as mean ± SD. $^{a}P < 0.05$ *vs* control.

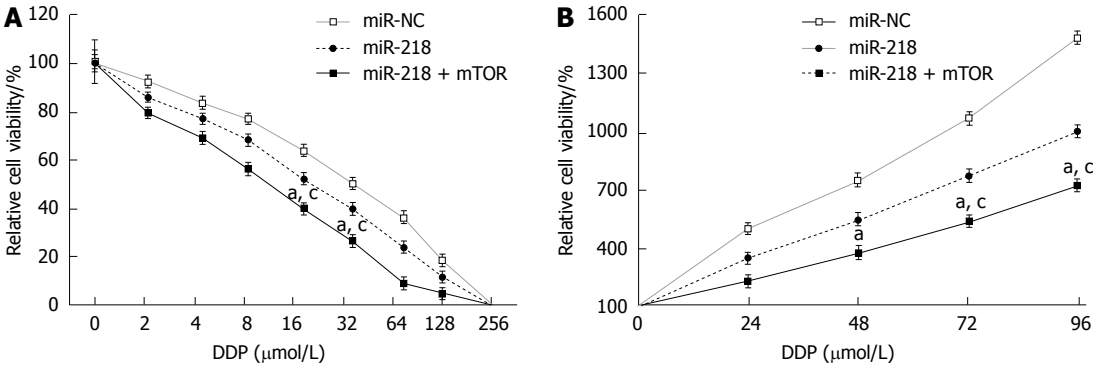

Figure 4 miR-218 increased chemosensitivity to cisplatin could be accelerated by overexpression of mTOR. Cells were transfected with miR-NC, or miR-218 with or without mTOR cDNA. Cells were exposed to cisplatin for further detection 10 h after transfection. A: Tumor cells proliferation assay of different cisplatin concentrations. 6 × 10^3 cells were seeded in 96-well plates and incubated with different concentration of cisplatin for 72 h. Data are presented as mean ± SD from three independent experiments performed in sextuple; B: Cell proliferation in the presence of 12 μmol/L cisplatin. Data are presented as mean ± SD from three independent experiments performed in sextuple. $^aP < 0.05$ *vs* control; $^cP < 0.05$ *vs* miR-218 + mTOR.

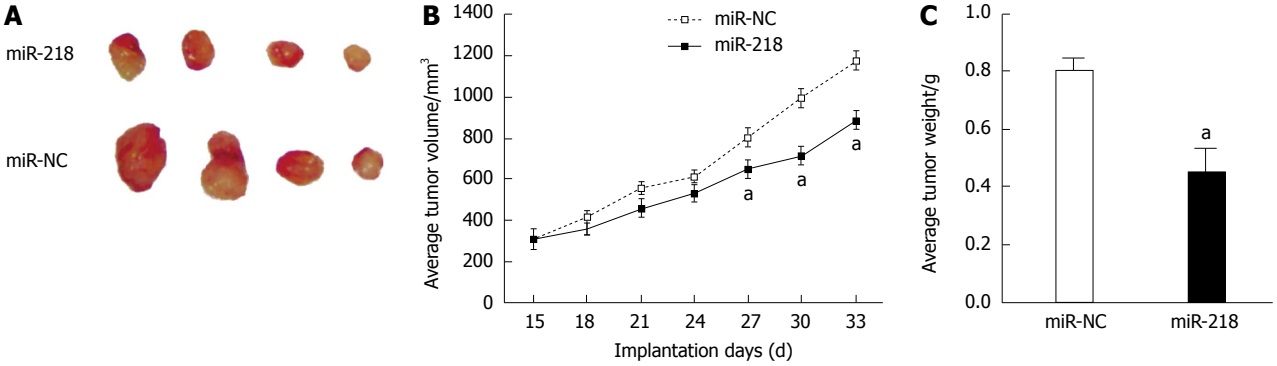

Figure 5 Upregulation of miR-218 inhibited gastric tumor growth *in vivo*. Cells stably expressing miR-218 or miR-NC were incubated in Dulbecco's modified Eagle's medium and subcutaneously injected into each side of the posterior flank of nude mice ($n = 24$). Thirty-three days after injection, mice were sacrificed and tumors were removed. A: Tumor volume at 33 d; B: Tumor volumes were detected every three days from the time they were obvious; C: Average tumor weights. [a]Denoted statistical significance between the two groups of miR-218 and control. $^aP < 0.05$ *vs* control.

tantly, differential miRNA expression patterns before and after CRS + HIPEC provide a solid basis for further validation, including functional studies to identify other potential oncogenic or tumor suppressor miRNAs in AGC.

COMMENTS

Background

Gastric cancer is the second leading cause of cancer death in the world, with often a poor prognosis due to the advanced stage of the disease at diagnosis.

Research frontiers

2010 French guidelines designated cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC) as the standard method for treating colorectal PCs, and evaluated the effectiveness of CRS + HIPEC treatment for other primary tumors, including gastric cancer, ovarian, and sarcoma.

Applications

To further define the function of miR-218, it was upregulated in human gastric cancer cells (SGC7901), and the cell vitality and chemosensitivity were then studied.

Peer review

This is an interesting study with both *in vivo* and *in vitro* data. The discovery of miR-218 was based on microarray results; hence the clinical significance is good.

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P- Reviewer: Chen RF, Fang Y, George V, Spisni E
S- Editor: Qi Y **L- Editor**: Rutherford A **E- Editor**: Wang CH

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DOI: 10.3748/wjg.v20.i32.11356

World J Gastroenterol 2014 August 28; 20(32): 11356-11362
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

RETROSPECTIVE STUDY

Effect of low-dose tacrolimus with mycophenolate mofetil on renal function following liver transplantation

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Supported by Grants from The National Sciences and Technology Major Project of China, No. 2012ZX10002-016 and 2012ZX10002-017
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Received: January 12, 2014 Revised: March 15, 2014
Accepted: April 21, 2014
Published online: August 28, 2014

Abstract

AIM: To determine whether low-dose tacrolimus (TAC) combined with mycophenolate mofetil (MMF) is a safe approach to decrease the incidence of chronic kidney disease (CKD) in liver transplantation (LT) recipients.

METHODS: We analyzed the medical records of 689 patients who underwent LT between March 1999 and December 2012 in a single Chinese center. Immunosuppression was initiated with a calcineurin inhibitor (TAC or CSA) and prednisone with or without MMF. CKD is defined by the glomerular filtration rate (GFR), estimated by an abbreviated Modification of Diet in Renal Disease formula, < 60 mL/min per 1.73 m^2 for at least 3 consecutive months after LT. Individuals with TAC trough concentrations ⩽ 8 ng/mL at 3 mo after LT were defined as the low-dose group. The incidence of CKD within 5 years was compared between the TAC group and the CSA group, as well as between four subgroups (low-dose and high-dose TAC groups with or without MMF).

RESULTS: No difference regarding the occurrence of pre-LT renal dysfunction or that of post-LT rejection was found between the TAC and CSA groups or between the four subgroups. With a definition of GFR < 60 mL/min per 1.73 m^2, the overall incidence of CKD was significantly higher in the CSA group than in the TAC group. The incidence of CKD in the low-dose TAC + MMF group (7.7%) was significantly lower than that observed in the low-dose TAC group (15.9%), high-dose TAC group (24.6%) and high-dose TAC + MMF group (18.5%). The cumulative 1-, 3- and 5-year incidence rates of CKD were 12.7%, 14.5% and 16.7%, respectively. The cumulative 5-year survival rates were 61.7% and 82.2% in patients with or without CKD, respectively.

CONCLUSION: In LT patients, the choice of immunosuppressive therapy appears to affect renal function and patient survival.

Key words: Liver transplantation; Chronic kidney disease; Calcineurin inhibitor; Mycophenolate mofetil

Core tip: Calcineurin inhibitor nephrotoxicity has been proposed to have a central role in chronic kidney disease, which has become a leading cause of long-term morbidity and mortality after liver transplantation. This study was conducted in 689 consecutive liver transplantation recipients and suggested that the choice of the immunosuppression therapy should be low-dose tacrolimus combined with mycophenolate mofetil, as this treatment was associated with better renal function and a higher patient survival rate.

Hao JC, Wang WT, Yan LN, Li B, Wen TF, Yang JY, Xu MQ, Zhao JC, Wei YG. Effect of low-dose tacrolimus with mycophe-

nolate mofetil on renal function following liver transplantation. *World J Gastroenterol* 2014; 20(32): 11356-11362 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11356.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11356

INTRODUCTION

The use of the calcineurin inhibitors (CNIs) cyclosporine A (CSA) and tacrolimus (TAC) in liver transplantation (LT) has greatly increased recipient survival rates and reduced graft rejection rates[1]. However, CNI nephrotoxicity has been proposed to have a central role in chronic kidney disease (CKD) after LT[2-4]. CKD has become a leading cause of long-term morbidity and mortality[5]. Ojo *et al*[5] reported that 18% of 36849 LT recipients had chronic renal dysfunction defined as a glomerular filtration rate (GFR) < 29 mL/min per square meter 5 years after LT.

Although the different impacts of TAC and CSA on renal function remains controversial, there has been substantial evidence that TAC has lower nephrotoxicity potential than CSA, especially in kidney transplant recipients. Numerous studies have shown that recipients treated with TAC display better renal function than those who receive CSA[6-9]. However, Alghamdi *et al*[10] found no difference in the mean levels of serum creatinine between the CSA and TAC groups, but this effect remains inconclusive in LT patients[11,12].

The most direct strategies to prevent CNI nephrotoxicity are avoidance and withdrawal of the drug. However, the exclusion of CNIs from immunosuppressive regimens does not preserve allograft function due to inadequate acute rejection prophylaxis by other immunosuppressive regimens[13-16]. Herein, novel CNI minimization protocols, in which the doses of cyclosporine or tacrolimus are adjusted to lower target levels, were conducted to balance potent immunosuppression and reduce CNI exposure. Morard *et al*[17] showed that independent risk factors associated with impaired renal function were trough levels of CSA ⩾ 150 ng/mL or TAC ⩾ 10 ng/mL at 1 year and CSA ⩾ 100 ng/mL or TAC ⩾ 8 ng/mL at 5 years. Our previous study showed that groups with ideal trough concentrations (CSA trough concentrations ⩽ 150 ng/mL, TAC trough concentrations ⩽ 8 ng/mL) had a significantly lower incidence of CKD[18]. A recent meta-analysis of 64 studies demonstrated that lower trough concentrations of TAC (6-10 ng/mL during the first month) had no significant influence on acute rejection and that TAC would be more appropriate after LT[19].

Immunosuppressive drugs without renal side effects have been increasingly used as CNI-sparing agents. Mycophenolate mofetil (MMF) is a non-nephrotoxic drug that inhibits the proliferation of T and B lymphocytes and has proven efficacy in the field of LT[20]. Substantial evidence has been found to suggest that MMF induction and maintenance used in conjunction with CNI following LT resulted in a significant improvement in renal function[21-25].

The aims of the present study were to determine whether TAC yields a lower incidence of CKD than CSA in recipients undergoing LT, as well as to investigate whether the use of reduced-dose TAC combined with MMF is a relatively safe approach to decrease the incidence of CKD.

MATERIALS AND METHODS

Study population

In this retrospective study, data from the clinical records of 940 consecutive patients who underwent LT between March 1999 and December 2012 in a single Chinese center were analyzed. The observation period ended on August 31, 2013, or at the time of patient death. Recipients who were followed up for less than 3 mo or died within 3 mo after transplantation, as well as those younger than 18 years, were excluded from the current study. All liver grafts were obtained from brain-dead or living donors. Living and deceased donations were voluntary in all cases, were approved by the West China Hospital Ethics Committee and were in accord with the ethical guidelines of the Declaration of Helsinki.

Evaluation of renal function

Renal function was assessed by the estimated GFR (eGFR) obtained using the abbreviated Modification of Diet in Renal Disease formula: eGFR = 186 × creatinine $(mg/dL)^{-1.154}$ × $(age)^{-0.203}$ × (0.742 if female)[26]. CKD was defined as having a GFR < 60 mL/min per 1.73 m^2 for at least 3 consecutive months after LT. Renal dysfunction before LT was also defined as having an eGFR < 60 mL/min per 1.73 m^2. Acute kidney injury (AKI) was defined as a > 25% decrease in GFR in the first post-operative week compared with the pre-operative level[27].

Definitions of other clinical parameters

Acute rejection (AR) and chronic rejection (CR) were confirmed by liver biopsy. Mayo end-stage liver disease scores were calculated for each patient.

Immunosuppressive protocols

Immunosuppression was initiated with a CNI (TAC or CSA) and prednisone with or without MMF. The initial dose of CNI was 0.05-0.10 mg/kg daily for TAC and 5-10 mg/kg daily for CSA. The dose of MMF was determined individually and ranged from 1.0-1.5 g/d. At our center, patients were only administered MMF during the early phase after transplantation when they were diagnosed with hypertension or diabetes mellitus; however, all recipients in the late post-transplant period were administered MMF unless severe gastrointestinal side effects or myelosuppression occurred. Prednisone was generally discontinued within 3 mo after transplantation.

Adjustment of calcineurin inhibitor dose during follow-up

CNI trough concentrations were checked daily for the first week, weekly for the next three weeks during the first post-operative month, monthly within 3 mo and ev-

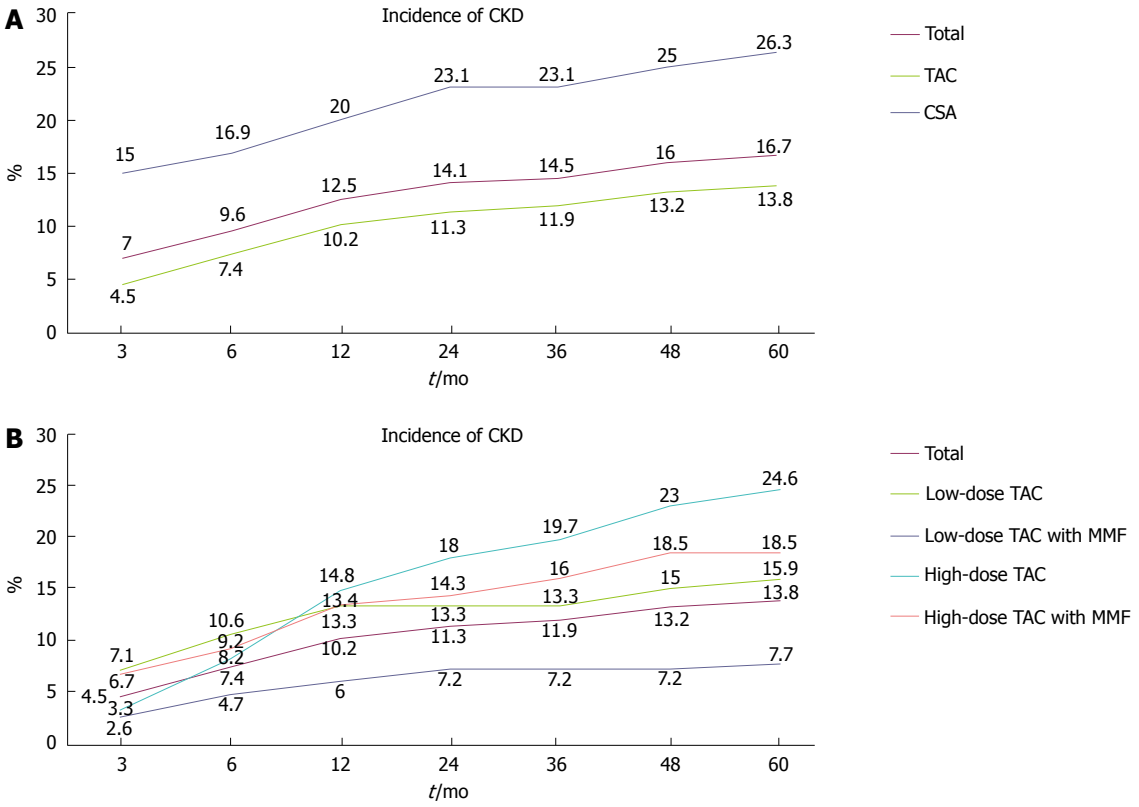

Figure 1 Incidence of chronic kidney disease. A: Incidence of chronic kidney disease (CKD) in the tacrolimus (TAC) and cyclosporine A (CSA) groups ($P < 0.05$); B: Incidence of CKD in the four TAC subgroups ($P < 0.05$). The estimated glomerular filtration rate (eGFR) was calculated by the abbreviated modification of diet in renal disease formula after each patient visit. Once the criterion for CKD (eGFR < 60 mL/min per 1.73 m^2) was met, the patient was registered in the CKD group.

ery 3 mo thereafter. The ideal serum trough level of CNI was 6-8 ng/mL for TAC and 120-150 ng/mL for CSA at 3 mo after operation. We classified patients with a TAC trough concentration ⩽ 8 ng/mL at 3 mo after LT as the low-dose group. Liver function was monitored intensely while adjusting the CNI dose. If AR occurred, the previous dosage was restarted with an increased prednisone dosage or high-dose methylprednisolone administration. Dose reduction was carefully and slowly carried out. A trough level of 4-6 ng/mL for TAC and 80-120 ng/mL for CSA one year after transplantation was expected to achieve stable liver function.

Statistical analysis

Numerical data are presented as the mean ± SD or as the median. The χ^2 test or Fisher's exact test was used to compare categorical variables. Continuous data were compared using the independent *t* test if data were normally distributed or using the rank-sum test if data were non-normally distributed. All analyses were performed using SPSS 22.0 statistical software (IBM Corporation, Armonk, NY). $P < 0.05$ was considered statistically significant.

RESULTS

Demographics

The medical records of 689 patients who met the inclusion criteria, including 575 males and 114 females with a mean age of 44.94 years, were retrospectively reviewed. The median follow-up duration was 24 (3-120) mo. The two most common primary diagnoses for recipients were tumors and cirrhosis, with 344 (49.9%) and 232 (33.7%) patients, respectively. More than 80% of cases were found to be hepatitis B virus (HBV)-related. The deceased donor transplantation rate was 74.2%. TAC group patients were divided into two groups based on the critical ideal trough concentration of ⩽ 8 ng/mL at 3 mo after transplantation. Furthermore, four subgroups were created depending on whether MMF therapy was adopted.

Incidence of chronic kidney disease

The eGFR was calculated after each patient visit. Once the criterion for CKD was met, the patient was registered in the CKD group. As shown in Figure 1, 16.7% of the entire patient population (115 cases) developed CKD during the 5-year follow-up.

No differences were found in renal dysfunction before LT or in AKI, AR and CR after LT between the TAC and CSA groups (Table 1) as well as between the four TAC subgroups (low-dose and high dose TAC with or without MMF, Table 2). The cumulative incidence of CKD at 3, 6, 12, 24, 36, 48 and 60 mo was 15.0%, 16.9%, 20.0%, 23.1%, 25.0% and 26.3%, respectively, in the CSA group and 4.5%, 7.4%, 10.2%, 11.3%, 11.9%, 13.2% and

Table 1 Baseline patient demographics and clinical characteristics in the tacrolimus and cyclosporine A groups *n* (%)

	Total (*n* = 689)	TAC (*n* = 529)	CSA (*n* = 160)	*P* value
Age (yr)	44.94 ± 9.68	44.70 ± 9.25	45.73 ± 10.98	NS
Gender (male/female)	575/114	448/81	127/33	NS
Donor type (DDLT/LDLT)	511/178	368/161	143/17	< 0.001
MELD score	13.77 ± 7.77	13.54 ± 7.96	14.54 ± 7.07	NS
Pre-LT renal dysfunction	61 (8.9)	46 (8.7)	15 (9.4)	NS
Post-LT AKI	108 (15.7)	80 (15.1)	28 (17.5)	NS
Post-LT AR	78 (11.3)	53 (10.0)	25 (15.6)	NS
Post-LT CR	18 (2.6)	13 (2.5)	5 (3.1)	NS
Primary diagnosis				NS
Tumors	344 (49.9)	265 (50.1)	79 (49.4)	
Cirrhosis	232 (33.7)	182 (34.4)	50 (31.3)	
Chronic active hepatitis	54 (7.8)	36 (6.8)	18 (11.3)	
Others	59 (8.6)	46 (8.7)	13 (8.1)	
Viral infection				NS
HBV infection	564 (81.9)	435 (82.2)	129 (80.6)	
HCV infection	9 (1.3)	8 (1.5)	1 (0.6)	

Age: Age at transplantation; LT: Liver transplantation; DDLT: Deceased donor liver transplantation; LDLT: Living donor liver transplantation; MELD: Mayo end-stage liver disease; AKI: Acute kidney injury; AR: Acute rejection; CR: Chronic rejection; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NS: No significance; TAC: Tacrolimus; CSA: Cyclosporine A.

Table 2 Baseline patient demographics and clinical characteristics in the tacrolimus group *n* (%)

	Total (*n* = 529)	Low-dose TAC (*n* = 113)	Low-dose TAC with MMF (*n* = 235)	High-dose TAC (*n* = 61)	High-dose TAC with MMF (*n* = 120)	*P* value
Age (yr)	44.00 ± 9.25	45.00 ± 9.61	44.86 ± 9.21	42.93 ± 8.83	44.98 ± 9.20	NS
Gender	448/81	95/18	200/35	51/10	102/18	NS
Donor type	368/161	102/11	143/92	49/12	74/46	< 0.001
MELD score	13.54 ± 7.96	15.03 ± 8.34	13.15 ± 7.85	13.48 ± 6.59	12.94 ± 8.37	NS
Renal dysfunction pre-LT	46 (8.7)	15 (13.3)	16 (6.8)	8 (13.1)	7 (5.8)	NS
Post-LT AKI	80 (15.1)	22 (19.5)	33 (14.0)	11 (18.0)	14 (11.7)	NS
Post-LT AR	53 (10.0)	15 (13.3)	18 (7.7)	10 (16.4)	10 (8.3)	NS
Post-LT CR	13 (2.5)	3 (2.7)	5 (2.1)	2 (3.2)	3 (2.5)	NS
Primary diagnosis						NS
Tumors	265 (50.1)	61 (54.0)	107 (45.5)	35 (57.4)	62 (51.7)	
Cirrhosis	182 (34.4)	39 (34.5)	89 (37.9)	17 (27.9)	37 (30.8)	
Chronic active hepatitis	36 (6.8)	9 (8.0)	12 (5.1)	4 (6.6)	11 (9.2)	
Others	46 (8.7)	4 (3.5)	27 (11.5)	5 (8.2)	10 (8.3)	
Viral infection						NS
HBV infection	435 (82.2)	95 (84.1)	191 (81.3)	48 (78.7)	101 (84.2)	
HCV infection	8 (1.5)	2 (1.8)	6 (2.6)	0	0	

Age: Age at transplantation; LT: Liver transplantation; DDLT: Deceased donor liver transplantation; LDLT: Living donor liver transplantation; MELD: Mayo end-stage liver disease; AKI: Acute kidney injury; AR: Acute rejection; CR: Chronic rejection; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NS: Not significance; TAC: Tacrolimus; CSA: Cyclosporine A.

13.8%, respectively, in the TAC group (Figure 1A, $P < 0.05$). The intragroup analysis of TAC showed that the low-dose TAC with MMF group had a significantly lower incidence of CKD at these times compared with the other three groups (2.6%, 4.7%, 6%, 7.2%, 7.2%, 7.2% and 7.7%, respectively; Figure 1B, $P < 0.05$).

The cumulative survival rates at 5 years after LT in patients with and without CKD were 61.7% and 82.2%, respectively (log-rank test, $P < 0.05$) (Figure 2).

DISCUSSION

The present study demonstrated a cumulative incidence of CKD of 12.5% within 1 year, 14.5% at 3 years and 16.7% at 5 years after LT. We observed that TAC supported better renal function than CSA, especially when used at a low dose and in combination with MMF. This combined regimen showed the most stable long-term outcome, with a CKD incidence of only 7.7% at 5 years after LT. We also confirmed that CKD yielded a lower patient survival rate.

All of the comparisons made before transplantation were similar, even if a difference appeared concerning donor type. These similarities were most likely due to the chronological aspect of the study. In our previous research, we confirmed that donor type had no significant impact on renal dysfunction[18].

There is substantial evidence that TAC has a lower nephrotoxicity potential than CSA. Animal studies have demonstrated that the vasoconstrictive effect of TAC is

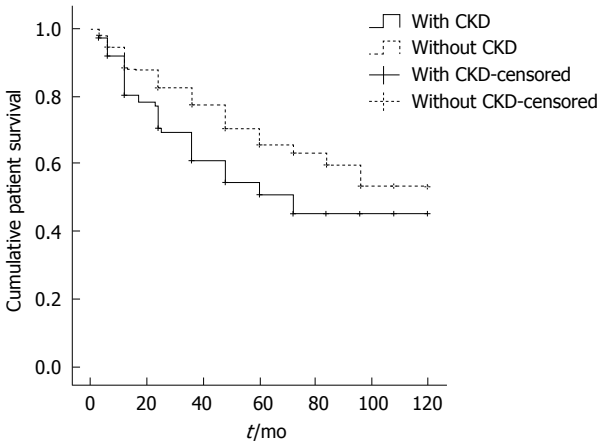

Figure 2 Kaplan-Meier analysis of cumulative patient survival in patients with and without chronic kidney disease. The cumulative survival was significantly higher in the non-chronic kidney disease (CKD) group (log-rank test, $P < 0.05$).

weaker than that of CSA[28-30], and this effect was also apparent in humans[31,32]. Moreover, the fibrogenic potential of TAC appears to be lower than that of CSA[33], although these results could not be confirmed in a more recent study[34]. For nonrenal solid organ transplantation, there have been single and multicenter studies, as well as registry analyses, that demonstrate the benefit of TAC over CSA with regard to renal function[5,35-37]. However, other studies exist that do not report this benefit of TAC over CSA[38].

In contrast to CSA, for which no dose-finding studies were performed before its introduction into clinical practice, extensive studies on TAC were performed before its introduction, and these demonstrated significant correlations between concentration and rejection and between concentration and toxicity[39]. To avoid CNI nephrotoxicity, minimizing CNI levels may be a better option, but it has become clear that the increased risk of allograft rejection could negate these positive effects. The positive effects observed following the introduction of MMF, which does not interfere with CNI actions or cause renal toxicity, as a rescue treatment for renal dysfunction due to CNI toxicity have been reported in several studies[40-42]. Moreover, MMF could have nephroprotective properties. Romero *et al*[43] noted that MMF prevented progressive renal failure in rats that underwent 5/6 renal ablation and hypothesized that MMF has an antiproliferative effect. In fact, in various cell lines (*e.g.*, smooth muscle cells, renal tubular cells and mesangial cells), MMF reduced or even abrogated proliferation in response to proliferative stimuli[44]. These same effects on endothelial cells may counteract the harmful vascular effects of CNI and explain the beneficial effects of MMF in the prevention and treatment of CNI toxicity apart from the effect of a CNI dose decrease. As a result, the lower decrease in the GFR observed in patients receiving MMF may not be due only to the CNI dose reduction.

In conclusion, we showed that in LT patients, the optimal calcineurin inhibitor is low-dose TAC combined with MMF, as this treatment was associated with a better long-term GFR (10 mL/min per 1.73 m^2), thereby decreasing renal toxicity, and a higher patient survival rate.

ACKNOWLEDGMENTS

We thank the Chinese Liver Transplant Registry (http://www.cltr.org) for providing the data.

COMMENTS

Background

Calcineurin inhibitors (CNI), since their introduction in the 1980s, have been the cornerstone of maintenance immunosuppressive regimens in liver transplantation. The use of CNI has substantially decreased the risk of acute rejection and improved short-term outcomes. However, CNI is associated with severe adverse effects, such as nephrotoxicity, dyslipidemia and hypertension especially in cyclosporine A (CSA). Recent studies showed that tacrolimus is preferred over CSA as an immunosuppressive agent. Low dose administration of immunosuppressive agents, such as tacrolimus, might reduce the risk of graft rejection, as well as cut down the cost of immunosuppressive therapy. However, correlation between the dosage of tacrolimus and the associated side effects and survival rates in liver transplant (LT) patients is largely unknown.

Research frontiers

Tacrolimus (TAC) has been proved with a less nephrotoxicity than CSA as well as mycophenolate mofetil (MMF), which have been used commonly in transplantations. In the area of prevention of chronic kidney disease (CKD) after LT, the research hotspot is how to balance the potent immunosuppression and less CNI exposure.

Innovations and breakthroughs

This article focused on developing and validating a low-dose tacrolimus combined with MMF as a better choice of immunosuppression. The study included 689 consecutive liver transplantation recipients. Glomerular filtration rate, estimated by an abbreviated modification of diet in renal disease formula, suggested CKD when it is lower than 60 mL/min per 1.73 m^2 for at least 3 consecutive months after LT. TAC trough concentrations $\leqslant$ 8 ng/mL at 3 mo after LT was defined as low-dose group. Incidence of CKD within 5 years was compared between TAC group and CSA group, as well as among four subgroups (low-dose and high-dose TAC groups with or without MMF). This study was with a high volume cohort and suggested that the choice of the immunosuppression should be low-dose TAC combined with MMF because it was associated with a better renal function and a higher patient survival rate.

Applications

The study results suggest that the choice of immunosuppressive therapy appears to affect renal function and patient survival in LT.

Terminology

Tacrolimus (trade name Prograf) is a product of the bacterium *Streptomyces tsukubaensis*. It is a macrolide lactone and acts by inhibiting calcineurin. The drug is used primarily in liver and kidney transplantations, although in some clinics it is used in heart, lung, and heart/lung transplantations. It binds to the immunophilin FKBP1A, followed by the binding of the complex to calcineurin and the inhibition of its phosphatase activity. In this way, it prevents the cell from transitioning from the G0 into G1 phase of the cell cycle. Tacrolimus is more potent than ciclosporin and has less pronounced side-effects. CKD, also known as chronic renal disease, is a progressive loss in renal function over a period of months or years. The symptoms of worsening kidney function are non-specific, and might include feeling generally unwell and experiencing a reduced appetite. Often, chronic kidney disease is diagnosed as a result of screening of people known to be at risk of kidney problems, such as those with high blood pressure or diabetes and those with a blood relative with chronic kidney disease. Chronic kidney disease may also be identified when it leads to one of its recognized complications, such as cardiovascular disease, anemia or pericarditis. It is differentiated from acute kidney disease in that the reduction in kidney function must be present for over 3 mo.

Peer review

This is a good retrospective study in which the authors analyzed the effect of low-dose tacrolimus with mycophenolate mofetil on the incidence of chronic kidney disease following liver transplantation. The results are interesting and

suggest that the choice of the immunosuppression appears to affect renal function and patient survival following LT.

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P- Reviewer: Mathis AS **S- Editor**: Gou SX **L- Editor**: Ma JY
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World Journal of Gastroenterology

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DOI: 10.3748/wjg.v20.i32.11363

World J Gastroenterol 2014 August 28; 20(32): 11363-11369
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

RETROSPECTIVE STUDY

"Minimizing tacrolimus" strategy and long-term survival after liver transplantation

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Supported by National S and T Major Program, No. 2012 ZX10002004; National Natural Science Foundation of China, No. 81373160 and No. 81302074

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Received: February 9, 2014 **Revised:** March 20, 2014
Accepted: April 21, 2014
Published online: August 28, 2014

Abstract

AIM: To investigate the effect of the "minimizing tacrolimus" strategy on long-term survival of patients after liver transplantation (LT).

METHODS: We conducted a retrospective study of 319 patients who received LT between January 2009 and December 2011 at the First Affiliated Hospital of Zhejiang University School of Medicine. Following elimination of ineligible patients, 235 patients were included in the study. The relationship between early tacrolimus (TAC) exposure and survival period was analyzed by Kaplan Meier curves. Adverse effects related to TAC were evaluated by the χ^2 test. Routine monitoring of blood TAC concentration (TC) was performed using the PRO-Trac™ II Tacrolimus Elisa Kit (Diasorin, United States).

RESULTS: Of 235 subjects enrolled in the study, 124 (52.8%) experienced adverse effects due to TAC. When evaluating mean TC, the survival time of patients with a mean TC < 5 ng/mL was significantly shorter than that in the other groups (911.3 ± 131.6 d *vs* 1381.1 ± 66.1 d, 911.3 ± 131.6 d *vs* 1327.3 ± 47.8 d, 911.3 ± 131.6 d *vs* 1343.2 ± 83.1 d, $P < 0.05$), while the survival times of patients with a mean TC of 5-7, 7-10 and 10-15 ng/mL were comparable. Adverse effects due to TAC in all four groups were not significantly different. When comparing the standard deviation (SD) of TC among the groups, the survival time of patients with a SD of 2-4 was significantly longer than that in the other groups (1388.8 ± 45.4 d *vs* 1029.6 ± 131.3 d, 1388.8 ± 45.4 d *vs* 1274.9 ± 57.0 d, $P < 0.05$), while in patients with a SD < 2 and SD > 4, the survival time was not statistically different. Adverse effects experienced in all three groups were not statistically different. In Cox regression analysis, male patients and those with a primary diagnosis of benign disease, mean TC > 5 ng/mL and TC SD 2-4 had better outcomes.

CONCLUSION: The early "minimizing tacrolimus" strategy with a mean TC of 5-10 ng/mL and SD of 2-4 was beneficial in terms of long-term survival after LT.

Key words: Tacrolimus; Liver transplantation; Outcome; Minimizing tacrolimus; Immunosuppressive drug

Core tip: Liver transplantation (LT) is a life-saving technique for patients with end-stage liver disease. Tacrolimus (TAC) is the cornerstone immunosuppressant for prevention of graft rejection in many LT centers. However, frequent TAC-related toxicities are observed

and these are a major concern in LT patients. Thus, the "minimizing tacrolimus" strategy after LT is very important, especially in the early phase.

Jia JJ, Lin BY, He JJ, Geng L, Kadel D, Wang L, Yu DD, Shen T, Yang Z, Ye YF, Zhou L, Zheng SS. "Minimizing tacrolimus" strategy and long-term survival after liver transplantation. *World J Gastroenterol* 2014; 20(32): 11363-11369 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11363.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11363

INTRODUCTION

Liver transplantation (LT) is a life-saving technique for patients with end-stage liver disease. The primary outcomes following LT are poor, however, improvements in surgical techniques and in donor liver protection, specifically new developments in immunosuppressive drugs have led to enhanced graft survival rates[1]. Tacrolimus (TAC), is a calcineurin inhibitor, and has become the cornerstone immunosuppressant in many LT centers in recent years[2]. Compared to cyclosporine, TAC plays a greater role in reducing acute cellular rejection (ACR) leading to better graft and overall survival in LT patients[3]. However, the narrow therapeutic index and greater pharmacokinetic variability of TAC necessitates routine monitoring of TAC blood concentration, especially in the early phase after LT[4,5]. In addition, TAC-related toxicity, mainly due to over immunosuppression, is frequent in liver transplant patients and is currently the main concern in these patients[6]. Therefore, better strategies to optimize TAC in allograft recipients are needed, these include the "minimizing tacrolimus" strategy, identification and validation of pharmacodynamic biomarkers, and direct drug measurement at the target sites, *i.e.*, allograft tissue[7] and lymphocytes[8,9].

The currently recommended target range for blood tacrolimus concentration (TC) after LT is 10-15 ng/mL during the first 4-6 wk, and then gradually decreasing to 5-10 ng/mL during long-term maintenance[10-14]. The higher TAC concentration, especially during the early phase after LT, may be associated with adverse effects such as hepatocellular carcinoma[15], early renal impairment[16,17] and even death[18]. Numerous studies used lowered early TC level known as the "minimizing tacrolimus" strategy to avoid these over immunosuppression side effects. A meta-analysis (n = 957) compared the standard dosage of TAC (10-15 ng/mL) with a minimized dosage (5-8 ng/mL) and showed no variation in biopsy-proven rejection over a 6-week follow-up period[19]. Early exposure to a TC of 7-10 ng/mL (the initial 2 wk) following LT has been proved to be safe with regards to acute rejection and longer graft survival[20]. Thus, an early TC of 5-7 ng/mL is safe for preventing rejection, however, the effect of this concentration on long-term survival is unknown. In this study, we introduced conventional standard deviation (SD) into the evaluation of early TC exposure, and attempted to provide evidence that a TC of 5-7 ng/mL is beneficial for the long-term survival of LT patients. We further explored the "minimizing tacrolimus" strategy on long-term survival of the patients after LT.

MATERIALS AND METHODS

Ethics statement

Ethical approval was obtained from the Committee of Ethics in Biomedical Research of Zhejiang University and all participants provided written informed consent forms. The design of this research was hospital-based and was approved by the China Liver Transplant Registry.

Patient population

In the present prospective study, 319 patients who received LT between January 2009 and December 2011 at the First Affiliated Hospital of Zhejiang University School of Medicine (Hangzhou, China) were enrolled. The entire research was based on prospectively collected data. Subjects who received a liver from an ABO incompatible donor (n = 67), underwent combined transplantation (n = 1), and had blood TC monitored less than 3 times in 4 wk were excluded from the study. After exclusion, 235 patients were enrolled in the study; 38 of these patients were considered to have clinical rejection and a liver biopsy was performed. Routine blood TC was assessed before the first daily dose of TAC was administered. Daily TC monitoring was performed using the PRO-Trac™ II Tacrolimus Elisa Kit (Diasorin, United States) following the manufacturer's instructions. In addition, alanine aminotransferase, aspartate aminotransferase, albumin, white blood cell, bilirubin, urea and creatinine levels were examined and the baseline clinical characteristics of the patients, such as gender, age, ABO group, MELD score, and Plug score were recorded.

The study groups were provided with TAC as the primary immunosuppressant following LT, which was administered with corticosteroids and mycophenolate mofetil (MMF). TAC was initiated at a daily dose of 2-3 mg, according to renal function, in post-LT patients for the first month and was titrated down to 5-10 ng/mL for the next few months. 1000 mg methylprednisolone was initiated in the perioperative phase, tapered slowly and withdrawn within the first month after LT. MMF was administered at 500 mg twice daily after LT.

Similar intra-operative and post-transplant care was provided to all patients. Piperacillin-tazobactam (4.5 g) three times a day (*tid*) was prescribed for postoperative antimicrobial prophylaxis for at least a week. Fluconazole (200 mg) per day was administrated for antifungal prophylaxis for two weeks and intravenous ganciclovir (5 mg/kg) per day was provided for antiviral prophylaxis for 2 wk. Patients with HBV received lamivudine plus hepatitis B immunoglobulin for post-transplant prophylaxis.

The parameters measured were survival period after

Table 1 Clinical characteristics of 235 consecutive liver transplant patients

Variables		Value
Age (yr)		47.11 ± 11.32
MELD score		16 (range 6-50)
Child score		9 (range 5-15)
Gender	Male	203 (86.4)
	Female	32 (13.6)
Etiology	HBV-related	140 (59.6)
	Hepatocarcinoma	55 (23.4)
	Others	40 (17)
Blood tests	Bilirubin (μmol/L) (NR < 17.1)	162.73 ± 205.39
	AST (IU/L) (NR < 50)	240.46 ± 556.16
	ALT (IU/L) (NR < 40)	183.2 ± 325.93
	Albumin (g/L) (NR 35-50)	34.96 ± 5.47
	WBC (× 10^9/L) (NR 3.5-11)	5.96 ± 4.58
	Urea (mmol/L) (NR 3-7.1)	6.70 ± 5.29
	Creatinine (μmol/L) (NR 60-97)	94.37 ± 68.98
TC		7.91 ± 2.28
ABO	A-A	85 (36.2)
	B-B	51 (21.7)
	O-O	87 (37)
	AB-AB	12 (5.1)

Data are expressed as absolute numbers (percentage) or mean ± SD. NR: Normal range; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; WBC: White blood cell; HBV: Hepatitis B virus.

Table 2 Clinical Fk506-related side effects and cause of death

	All events (*n*)	First events only (*n*)
Rejection	38	20
Infection	55	26
Renal insufficiency	7	2
Tumor recurrence	36	18
Metabolic disorder	34	21
Cause of death		
MODS	11	
Tumor recurrence	22	
Graft loss	3	
Others	11	

MODS: Multiple organ dysfunction syndrome.

LT, blood TC, number of patients with graft rejection, renal insufficiency, infection, metabolic disorders and tumor recurrence. The median follow-up time was 872 d. Renal insufficiency was defined as serum creatinine ⩾ 130 μmol/L, lasting at least 1 mo after LT.

Statistical analysis

The sample size estimation was based on previous logistic and Cox regression analyses of clinical trials[16]. Statistical analysis was performed using SPSS 17.0 (Chicago, IL, United States). Variables were displayed in frequency tables or expressed as means and standard deviations, or as medians and interquartile range. The χ^2 test was used for comparison of frequencies and the optimal threshold value for TC (mean or standard deviation, SD) related to long-term survival was established by receiver operating characteristic curves or previous publications. Kaplan Meier curves and Cox regression analysis were used to determine the influence of early TC (mean or SD) on long-term outcomes. Each hypothesis tested was two tailed and significant if $P < 0.05$.

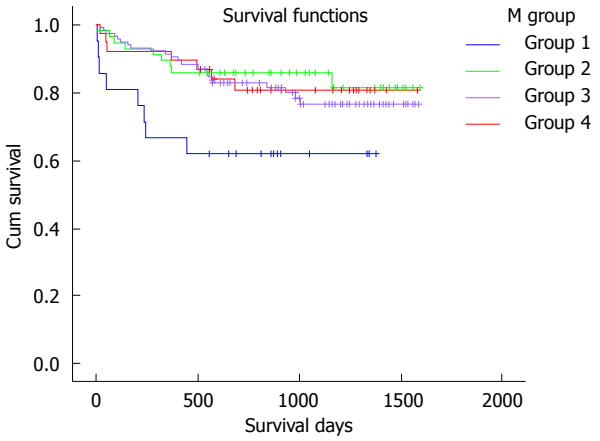

Figure 1 Patient survival curve according to the mean tacrolimus concentration. The survival time of group 1 is obviously shorter than other groups (group 1 *vs* group 2, 911.3 ± 131.6 d *vs* 1381.1 ± 66.1 d, $P = 0.018$; group 1 *vs* group 3, 911.3 ± 131.6 d *vs* 1327.3 ± 47.8 d, $P = 0.024$; group 1 *vs* group 4, 911.3 ± 131.6 d *vs* 1343.2 ± 83.1 d, $P = 0.067$) while the rates in groups 2, 3 and 4 are almost the same (group 2 *vs* group 3, 1381.1 ± 66.1 d *vs* 1327.3 ± 47.8 d, $P = 0.501$; group 2 *vs* group 4, 1381.1 ± 66.1 d *vs* 1343.2 ± 83.1 d, $P = 0.774$; group 3 *vs* group 4, 1327.3 ± 47.8 d *vs* 1343.2 ± 83.1 d, $P = 0.801$). Group 1: Mean tacrolimus concentration is 0-5 ng/mL, Group 2: 5-7 ng/mL, Group 3: 7-10 ng/mL, and Group 4: 10-15 ng/mL.

RESULTS

Study description

The pre-transplant diagnosis and the demographics of the patient population are listed in Table 1. In total, 235 subjects were enrolled, 59.6% of the subjects had hepatitis B-related disease (severe hepatitis or cirrhosis), 23.4% had hepatocarcinoma and 17% had other diseases (alcoholic cirrhosis in 15 cases, primary biliary cirrhosis in 8 cases, hepatolenticular degeneration in 3 cases, cholangiocarcinoma in 3 cases, diffuse biliary calculi in 3 cases, schistosoma-related cirrhosis in 3 cases, hepatitis C in 2 cases, glycogen storage disease in 2 cases, and hepatic veno-occlusive disease in 1 case). Of the 235 subjects, 47 patients (20%) died within the observation period and 124 patients (52.8%) experienced at least one TAC-related side effect (Table 2).

Relationship between mean TC and patient outcome

Patients were divided into four groups according to the mean TC intervals during the first four weeks after LT: 21 (8.9%) cases had a mean TC < 5 ng/mL (group 1), 57 (24.3%) cases had a mean TC of 5-7 ng/mL (group 2), 119 (50.6%) cases had a mean TC of 7-10 ng/mL (group 3) and 38 (16.2%) cases had a mean TC of 10-15 ng/mL (group 4). The survival curves of these groups are shown in Figure 1. TAC-related side effects observed during follow-up in each group are listed in Table 3. Pairwise comparisons between the groups were not statistically different ($P > 0.05$).

Table 3 Classification of groups according to mean tacrolimus concentration, death rate and related side effects in respective categories

Group	1	2	3	4
TC interval (ng/mL)	0-5	5-7	7-10	10-15
Total number	21	57	119	38
Number of death	8	9	23	7
Cause of death				
MODS	4	4	2	1
Tumor recurrence	2	4	13	3
Graft loss	1	1	0	1
Others	1	0	8	2
Rejection	2	14	16	6
Infection	6	14	24	11
Renal insufficiency	2	2	2	1
Tumor recurrence	5	7	19	5
Metabolic disorder	6	8	15	5

MODS: Multiple organ dysfunction syndrome; TC: Tacrolimus concentration.

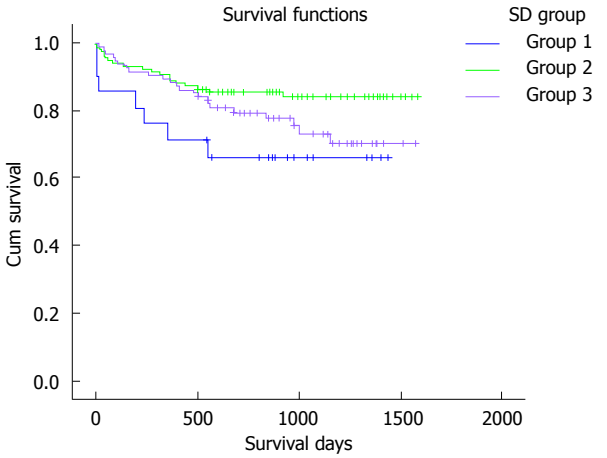

Figure 2 Patient survival curve according to the standard deviation of tacrolimus concentration. The survival time of group 2 is obviously longer than that of other groups (group 1 *vs* group 2, 1029.6 ± 131.3 d *vs* 1388.8 ± 45.4 d, $P = 0.032$; group 3 *vs* group 2, 1274.9 ± 57.0 d *vs* 1388.8 ± 45.4 d, $P = 0.044$) while in groups 1 and 3, the rates are not statistically different (1029.6 ± 131.3 d *vs* 1274.9 ± 57.0 d, $P = 0.286$). Note: Group 1: Standard deviation (SD) of tacrolimus concentration was 0-2 ng/mL, Group 2: 2-4 ng/mL, and Group 3: > 4 ng/mL.

Relationship between the SD of TC and patient outcome

The patients were divided into three groups according to the SD of TC intervals during the first four weeks after LT: 21 (8.9%) cases had a SD < 2 (group 1), 118 (50.2%) cases had a SD of 2-4 (group 2) and 96 (40.9%) cases had a SD > 4 (group 3). The survival curve is shown in Figure 2. TAC-related side effects during the follow-up period are listed in Table 4. Pairwise comparisons between the groups were not statistically different ($P > 0.05$).

Cox regression analysis

Predictive factors, such as gender, age, primary diagnosis, Plug score, MELD score, mean TC, and TC SD were analyzed first by a univariate, then by a multivariate Cox regression model. In the univariate Cox regression model, gender (female, $P = 0.041$), diagnosis (malignancy, $P = 0.03$), Plug score (> 10, $P = 0.042$), mean TC (< 5 ng/mL, $P = 0.013$) and TC SD (< 2 or > 4, $P = 0.049$) demonstrated statistical significance, while in the multivariate model, gender (female, $P = 0.022$), diagnosis (malignancy, $P = 0.03$), mean TC (< 5 ng/mL, $P = 0.016$) and TC SD (< 2 or > 4, $P = 0.024$) showed statistical significance in predicting overall survival after LT (Table 5). Therefore, male patients, those with a benign primary diagnosis, mean TC > 5 ng/mL and a TC SD of 2-4 showed a survival benefit.

Table 4 Classification of groups according to standard deviation of Tacrolimus concentration, death rate and related side effects in respective categories

Group	1	2	3
SD interval	0-2	2-4	> 4
Total number	21	118	96
Number of death	7	18	22
Cause of death			
MODS	4	4	3
Tumor recurrence	2	7	13
Graft loss	0	2	1
Others	1	5	5
Rejection	1	21	16
Infection	5	25	25
Renal insufficiency	0	6	1
Tumor recurrence	3	14	19
Metabolic disorder	2	20	12

MODS: Multiple organ dysfunction syndrome; SD: Standard deviation.

Table 5 Variables in multivariate Cox regression equation of long-term survival

	B	SE	Wald	*df*	Sig.	Exp(*B*)	Lower	Upper
Gender	-0.809	0.352	5.284	1	0.022	0.445	0.224	0.888
Age	0.252	0.612	0.170	1	0.680	1.287	0.388	4.273
Plug score	0.640	0.364	3.093	1	0.079	1.897	0.929	3.870
Meld score	-0.281	0.266	1.113	1	0.291	0.755	0.448	1.272
Diagnosis	-0.351	0.162	4.691	1	0.030	0.704	0.512	0.967
SD of TC	0.345	0.153	5.063	1	0.024	1.412	1.045	1.907
Mean TC	-0.960	0.397	5.830	1	0.016	0.383	0.176	0.835

SD: Standard deviation; TC: Tacrolimus concentration.

Evaluation of survival rate in selected patients

Patients were divided into two groups according to the selected mean TC and SD intervals during the first four weeks after LT: 107 (50.2%) patients had a mean TC > 5 ng/mL and SD of 2-4 (group 0), and 106 (49.8%) patients had a mean TC > 5 ng/mL and SD < 2 or SD > 4 (group 1). The survival curve is shown in Figure 3. The 3-year overall survival of group 0 was over 80% and was approximately 70% in group 1.

DISCUSSION

TAC, a calcineurin inhibitor, first developed by Starzl[21], is the foundation of conventional immunosuppression in LT recipients, and is preferable to cyclosporine as demonstrated in numerous randomized studies[3,22]. As

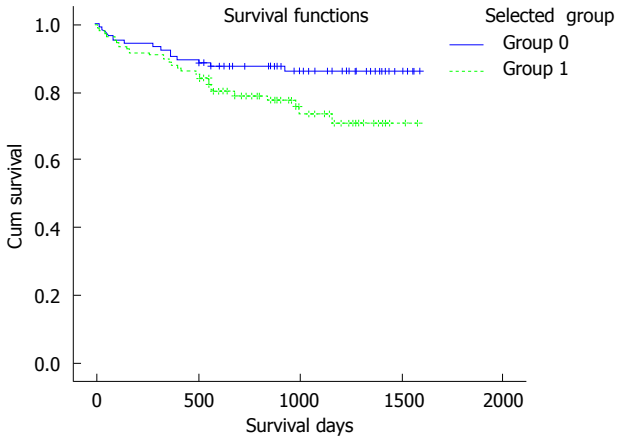

Figure 3 Patient survival curve according to the selected mean tacrolimus concentration and standard deviation. The survival time in group 0 is obviously longer than in group 1 (group 0 *vs* group 1, 1418.8 ± 44.5 d *vs* 1275.3 ± 53.7 d, $P = 0.043$). Note: Group 0: mean tacrolimus concentration (TC) > 5 ng/mL and standard deviation 2-4, Group 1: mean TC > 5 ng/mL and SD < 2 or > 4.

mentioned previously, TAC has unwanted side effects which are a significant issue in clinical practice. To reduce TAC-related side effects, considerable efforts should be made to offer patients the "minimizing tacrolimus" strategy, and early exposure to TC between 5 and 10 ng/mL which has already been confirmed to prevent acute rejection. Within our cohort of patients given TAC-based regimens, mean TC > 5 ng/mL during the initial four weeks following LT had almost the same survival curve as that for 10-15 ng/mL (the current recommended target range) without increasing graft rejection, which is consistent with previous data[19]. In addition, SD is yet another productive and important index in judging TC level. High SD in serial TC levels is related to an increased likelihood of late rejection and graft loss in pediatric organ transplant recipients[23]. Within our clinical setting, SD of 2-4 was shown to be the optimal SD for long-term survival, SD > 4 (in accordance with other reports) and SD < 2 (may due to the small number of patients (n = 21) or insensitivity to TAC) were both associated with a low patient survival rate. Additionally, Cox regression analysis showed that male gender, Plug score (< 10), primary diagnosis of benign disease, mean TC > 5 ng/mL and TC SD of 2-4 are independent factors in the long-term survival of patients, further confirming our results. Chronic TAC exposure may lead to numerous adverse reactions including infection, renal dysfunction, tumor recurrence, rejection, and metabolic disorders[17], and low TC was reported to be linked to rejection[24-26]. In our study, low early exposure to TAC was not associated with these adverse reactions or rejection, and this was mainly due to the brief exposure time which was not continuous or protracted; the entire population attained exactly the same target concentration of 5-10 ng/mL during maintenance treatment for 4-6 wk following LT.

This is the first time that the indices, mean TC and SD, to determine the effect of early TAC exposure on long-term survival have been incorporated, and it was found that the inclusion of mean TC > 5 ng/mL and SD of 2-4 in the optimal early exposure of TAC after LT led to an overall 3-year survival of over 80%. In our study the "minimizing tacrolimus" strategy showed the same rate of survival as the advised level of 10-15 ng/mL and did not increase the incidence of adverse reactions or graft rejection. Thus, this "minimizing tacrolimus" strategy is safe and practical for LT patients. However, it is clear that the "minimizing tacrolimus" strategy should be included with suitable scientific analysis of every patient, integrating clinical examination, biochemical evaluation and pathological analysis, in addition to the pharmacodynamics and pharmacokinetics of TAC.

In conclusion, there are no increased risks when the TC level is reduced compared with the currently recommended level (10-15 ng/mL) during the early phase after LT. We combined the mean and SD to evaluate TAC exposure and suggest that mean TC of 5-10 ng/mL and SD of 2-4 in the initial four weeks following LT are optimal for long-term survival. This "minimizing tacrolimus" strategy protects against unwanted effects, particularly graft rejection. However, further data are needed to support this strategy in order to confirm the results of this study.

COMMENTS

Background

Liver transplantation (LT) is a life-saving technique for patients with end-stage liver disease. Tacrolimus (TAC) is the cornerstone immunosuppressant used to prevent graft rejection in many LT centers. However, frequent TAC-related toxicities are observed and these are a major concern in LT patients. A high blood TAC concentration, especially during the early phase after LT, is associated with adverse effects such as hepatocellular carcinoma, early renal impairment and even death. Thus, the "minimizing tacrolimus" strategy is very important for better long-term outcome, especially in the early phase.

Research frontiers

The primary outcomes of liver transplantation are poor, however, improvements in surgical techniques and in donor liver protection, specifically new developments in immunosuppressive drugs, have led to enhanced graft survival rates. Tacrolimus is one of the immunosuppressant drugs used to prevent graft rejection in many LT centers. Due to TAC-related toxicity, numerous studies have demonstrated that lower early TAC concentration (TC) level also known as the "minimizing tacrolimus" strategy avoids these over immunosuppression side effects.

Innovations and breakthroughs

The parameter measured during tacrolimus exposure in previous studies has mainly been mean concentration. In this study, the authors combined mean TC and standard deviation (SD) to determine the effect of early TAC exposure on long-term survival and found that a mean TC > 5 ng/mL and SD of 2-4 were optimal levels for early TAC exposure in LT patients, which is lower than the currently recommended level of 10-15 ng/mL.

Applications

This study showed that there are no increased risks when the TC level is reduced below the currently recommended level (10-15 ng/mL). The authors recommend that mean TC of 5-10 ng/mL and SD of 2-4 during the initial four weeks following LT are optimal for long-term survival, and this "minimizing tacrolimus" strategy prevents unwanted effects and graft rejection. Therefore, early TAC exposure could be reduced to 5-10 ng/mL in clinical practice and the outcome of patients may be predicted according to the early mean TC and SD.

Terminology

"Minimizing tacrolimus" means lowering the tacrolimus dosage to avoid related toxicity and prevent graft rejection.

Peer review

This study introduced a novel method to evaluate the low level of TC by mean and SD. The authors report the outcomes of a prospective study of tacrolimus mean levels and SD in patients undergoing liver transplantation. The data of the complication rates reported at different tacrolimus levels is useful, as it is the identification of an optimal level of tacrolimus for maintenance.

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P- Reviewer: Boucek C, Wang GY **S- Editor**: Gou SX
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DOI: 10.3748/wjg.v20.i32.11370

World J Gastroenterol 2014 August 28; 20(32): 11370-11375
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

RETROSPECTIVE STUDY

Gastrointestinal symptoms: A comparison between patients undergoing peritoneal dialysis and hemodialysis

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Received: February 20, 2014 Revised: April 28, 2014
Accepted: July 11, 2014
Published online: August 28, 2014

Abstract

AIM: To compare the prevalence and diversity of gastrointestinal (GI) symptoms in patients undergoing peritoneal dialysis (PD) and hemodialysis (HD).

METHODS: Two hundred and ninety-four end-stage renal disease patients participated in the study, including 182 HD and 112 PD patients. Dimension scores were calculated from a modified gastrointestinal symptom rating scale (GSRS) 18-item questionnaire, including items concerning eating dysfunction, and were used for measuring GI symptoms. Information on patient age, condition contributing to end-stage renal disease and the most recent dialysis adequacy assessment (serum *Kt/V* urea value) was obtained from the follow-up database and by interviewing patients and/or reviewing the medical records. Differences between the HD and PD groups were evaluated using Student's *t*, Pearson's χ^2 or Fisher's exact tests.

RESULTS: The overall prevalence of GI symptoms, defined by a GSRS > 1, in end-stage renal disease patients was 70.7% (208/294), which differed between HD and PD patients (76.4% *vs* 61.6%, $P < 0.01$). HD patients had a higher prevalence of constipation, abdominal pain and diarrhea compared to PD patients (36.3% *vs* 17.9%, 32.4% *vs* 5.4%, 17.6% *vs* 4.5%, respectively, $P < 0.05$). PD patients had a higher prevalence of reflux compared to HD patients (32.1% *vs* 24.2%, $P < 0.05$). Additionally, reflux and eating dysfunction were more severe in PD patients (GSRS: 1.71 ± 1.15 *vs* 1.30 ± 0.67, 1.57 ± 0.84 *vs* 1.39 ± 0.61, respectively, $P < 0.05$), whereas HD patients had greater abdominal pain, diarrhea and constipation (GSRS: 1.22 ± 0.39 *vs* 1.04 ± 0.19, 1.19 ± 0.53 *vs* 1.07 ± 0.35, 1.51 ± 0.83 *vs* 1.23 ± 0.58, respectively, $P < 0.05$). Finally, 14.8% (27/182) of HD patients presented with more than three GI symptoms, compared to 7.2% (8/112) of PD patients ($P < 0.01$).

CONCLUSION: HD and PD patients differ in prevalence, severity and diversity of GI symptoms.

Key words: Gastrointestinal symptom; Hemodialysis; Peritoneal dialysis; End-stage renal disease; Constipation; Reflux; Eating dysfunction; Abdominal pain; Diarrhea; Indigestion

Core tip: End-stage renal disease patients undergoing dialysis frequently experience gastrointestinal symptoms. In agreement with previous studies, a majority of patients undergoing hemodialysis (HD) and peritoneal dialysis (PD) in the present study reported gastrointestinal symptoms. However, the results indicate that the prevalence and severity of various gastrointestinal symptoms differ between patients undergoing these two dialysis treatments. HD patients had a higher prevalence of and more severe constipation, abdominal pain and diarrhea, whereas PD patients experienced stronger and more frequent reflux and eating dysfunc-

tion. Furthermore, a significantly greater number of HD patients presented with more than three gastrointestinal symptoms.

Dong R, Guo ZY, Ding JR, Zhou YY, Wu H. Gastrointestinal symptoms: A comparison between patients undergoing peritoneal dialysis and hemodialysis. *World J Gastroenterol* 2014; 20(32): 11370-11375 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11370.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11370

INTRODUCTION

Gastrointestinal (GI) disorders are a common occurrence in the general population and significantly impair quality of life[1]. Furthermore, GI symptoms are common among patients with end-stage renal disease (ESRD)[2-5] and occur in 32%-85% of patients undergoing dialysis[6-8]. The incidence of GI symptoms can largely be attributed to the underlying conditions, such as increased level of uremic toxin, the effect of dialysis, lifestyle change, or the medications required for treatment[6,7]. Among patients undergoing regular hemodialysis (HD), 51.0%-70.7% experience some GI symptoms, which is significantly higher than that of controls[8,9] but lower than the reported 85% of peritoneal dialysis (PD) patients[8].

GI symptoms in PD patients most commonly include those of gastroesophageal reflux, dyspepsia and eating dysfunction[6,10]. Although PD patients are reportedly more likely than HD and pre-dialysis patients to suffer from these symptoms[6,7,11], few studies have directly or comprehensively evaluated these differences. Indeed, the increased prevalence of GI symptoms in PD patients remains controversial. Therefore, this study aimed to investigate the differences in the prevalence and diversity of GI symptoms between ESRD patients undergoing PD and those undergoing HD.

MATERIALS AND METHODS

Participants

Active PD and HD patients were recruited from the Blood Purification Center at the Changhai Hospital (Shanghai, China), including both inpatients and outpatients who had been receiving dialysis for at least three months. Patients with dementia, severe infectious illness, hepatocholecystopathy, peritonitis in the last three months, unstable blood pressure or glucose levels, or unwillingness to participate in the study were excluded. Informed consent was obtained from all patients in the study, which was approved by the Ethics Committee of Changhai Hospital.

Rating of gastrointestinal symptoms

Participants were asked to complete a modified gastrointestinal symptom rating scale (GSRS) questionnaire to evaluate the presence and severity of general GI symptoms during the previous 2 wk. The GSRS is a 15-item questionnaire with a 7-grade Likert scale (1 = none, 2 = minor, 3 = mild, 4 = moderate, 5 = moderately severe, 6 = severe, and 7 = very severe discomfort) that was originally constructed as an interview-based rating scale to evaluate a wide range of GI symptoms[12] and later modified to become a self-administered questionnaire[13]. The items are grouped into five dimensions, including abdominal pain (three items), reflux (two items), indigestion (four items), diarrhea (three items), and constipation (three items) syndromes. In addition, an eating dysfunction dimension was included, concerning early satiety, difficulties in eating normal portions and postprandial pain, which was developed in a manner analogous to the GSRS[14]. A dimension score was calculated as the mean value of the items belonging to the specific syndrome with a minimum value of 1 and a maximum value of 7.

Patient information

Information concerning age, disease leading to ESRD, diabetic status, and duration of dialysis was obtained by interviewing patients and/or reviewing the medical records. The most recent serum Kt/V urea, an index of dialysis adequacy, was obtained from the follow-up database. Kt/V was calculated using the Daugirdas formula[15].

Statistical analysis

Analyses were performed with SPSS for Windows, version 19.0 (IBM, Armonk, NY, United States). Student's *t*-tests were used to compare continuous variables between HD and PD patients when appropriate, and Pearson's χ^2 or Fisher's exact tests were used for categorical variables. Data are presented as the mean and standard deviation for continuous variables that are normally distributed, and as percentages for categorical variables. A P value < 0.05 was considered statistically significant.

RESULTS

Patient characteristics

In total, two hundred and ninety-four ESRD patients participated in the study and completed the questionnaires, including 182 HD patients and 112 PD patients. Patients undergoing HD and PD did not differ with regard to age, sex, the presence of diabetes mellitus, or the mean dialysis duration (Table 1). The most common cause of ESRD was chronic glomerular nephritis, followed by hypertensive nephropathy and diabetic nephropathy. Dialysis efficacies did not differ between the groups, with 66.7% (62/93) of PD patients able to keep their total (renal + peritoneal) Kt/V above the recommended 1.7[16], and 73.7% (132/179) of HD patients able to achieve a delivered Kt/V value of more than 1.2[17].

Prevalence of GI symptoms in the HD and PD groups

In total, the overall prevalence of GI symptoms (GSRS > 1) in ESRD patients was 70.7% (208/294). A significantly

Table 1 Clinical features of the study population *n* (%)

Clinical feature	HD (*n* = 182)	PD (*n* = 112)	*P* value
Age (yr)	58.67 ± 14.39	59.67 ± 14.19	0.56
Female	75 (41.2)	51 (45.5)	0.47
Diabetes mellitus	37 (20.3)	27 (24.1)	0.47
Disease leading to chronic renal failure			
Chronic glomerular nephritis	85 (46.7)	40 (35.7)	
Hypertensive nephropathy	38 (20.9)	34 (30.4)	
Diabetic nephropathy	27 (14.8)	22 (19.6)	
Polycystic kidney disease	9 (4.9)	7 (6.2)	
Gout	5 (2.7)	3 (2.7)	
Others	18 (9.0)	6 (5.4)	
Duration of dialysis, mean months	55.54 ± 38.47	48.90 ± 31.01	0.11
Kt/*V* target reached[1]	132 (73.7)	62 (66.7)	0.26

[1]Restricted to the 179 HD and 93 PD patients for whom complete information on *Kt*/*V* was available. HD: Hemodialysis; PD: Peritoneal dialysis.

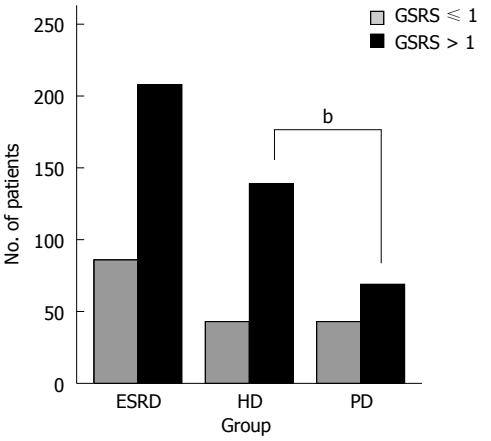

Figure 1 Gastrointestinal symptom rating scale scores. Numbers of end-stage renal disease (ESRD), hemodialysis (HD) and peritoneal dialysis (PD) patients with Gastrointestinal Symptom Rating Scale (GSRS) scores ≤ or > 1. [b]*P* < 0.01, HD *vs* PD.

larger number of patients in the HD group had a GSRS > 1 (139/182; 76.4%), compared to 61.6% (69/112) of patients in the PD group ($P < 0.01$) (Figure 1). In the HD group, more patients suffered from constipation, abdominal pain and diarrhea (36.3%, 32.4% and 17.6%, respectively), compared with those in the PD group (17.9%, 5.4% and 4.5%, respectively; $P < 0.01$ for all) (Figure 2). Although both groups show similar prevalences of indigestion and eating dysfunction, PD patients had a higher prevalence of reflux symptoms than HD patients (32.1% *vs* 24.2%, $P < 0.05$).

GSRS scores in the HD and PD groups

GSRS scores were significantly different between HD and PD patients in all dimensions except for indigestion ($P < 0.05$) (Table 2). Rating scores for abdominal pain, diarrhea and constipation were higher in HD patients, whereas PD patients reported more severe reflux and eating dysfunction.

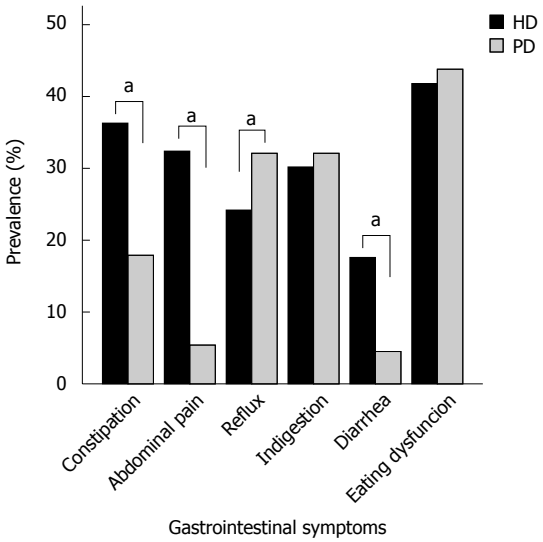

Figure 2 Prevalence of gastrointestinal symptoms. The prevalence of various gastrointestinal symptoms in hemodialysis (HD) and peritoneal dialysis (PD) patients. [a]*P* < 0.05, HD *vs* PD.

Diversity of GI symptoms in the HD and PD groups

The majority of the studied population had at least one reported GI symptom. Although most patients reported one to three symptoms, 27 HD patients reported experiencing four or more symptoms, compared to only eight of the PD patients (Table 3). There was a significantly different distribution of the number of GI symptoms between these two groups ($P < 0.01$).

DISCUSSION

The present study indicates that GI symptoms are common in dialysis patients, with an overall prevalence of 70.7%, similar to a previous report on chronic kidney disease patients[18]. However, these symptoms are more prevalent in HD than in PD patients. Previous studies have reported inconsistent prevalences of GI symptoms, ranging from 32% to 79% in HD patients[4,9,19,20] and 42% to 62% in PD patients[7,10,21], and 73.6% of patients with continuous ambulatory peritoneal dialysis (CAPD) had abnormal upper gastrointestinal endoscopic findings[22]. However, our results indicate a higher prevalence among HD patients, despite similar sample sizes. Such discrepancies may be attributed to the differences in questionnaires used.

The results of this study suggest that the severity of GI symptoms was quite different between HD and PD patients. A greater number of HD patients complained of abdominal pain, diarrhea and constipation, in agreement with previous observations[2,20,23]. The abdominal pain may be associated with peptic ulcers, as Chachati *et al*[24] observed a high incidence of upper GI pathology, including ulcers, during the first two years of HD, which declined with HD duration. A similar finding was reported in another study where peptic ulcers were shown to be less likely to recur in patients receiving HD for prolonged

Table 2 Comparison of gastrointestinal symptom rating scale scores between the two groups

Dimension	HD (n = 182)	PD (n = 112)	P value
Abdominal pain	1.22 ± 0.39	1.04 ± 0.19	0.000
Reflux	1.30 ± 0.67	1.71 ± 1.15	0.001
Indigestion	1.23 ± 0.47	1.32 ± 0.56	0.153
Diarrhea	1.19 ± 0.53	1.07 ± 0.35	0.019
Constipation	1.51 ± 0.83	1.23 ± 0.58	0.001
Eating dysfunction	1.39 ± 0.61	1.57 ± 0.84	0.049

HD: Hemodialysis; PD: Peritoneal dialysis.

Table 3 Number of gastrointestinal symptoms according to the gastrointestinal symptom rating scale

Group	No. of symptoms			P value[1]
	0	1-3	4-5	
HD	43	112	27	0.009
PD	43	61	8	

[1]Pearson's χ^2 analysis. HD: Hemodialysis; PD: Peritoneal dialysis.

therapy durations[25]. It is possible that HD patients are more susceptible to ischemic colitis due to hypotensive episodes, which are likely to occur during the initial stages of dialysis[21]. The prevalence of diarrhea in the HD patients of the current study was similar to results from a study by Cano *et al*[2] who used a Rome II questionnaire to evaluate GI symptoms. However, another previous study reported a much lower incidence, though that study found a gender difference, with women reporting more detrimental symptoms[23]. However, constipation was the most common and severe presenting GI symptom among HD patients, similar to findings from Yasuda *et al*[26] showing that constipation was more than three times more prevalent in HD compared to CAPD patients. In the clinic, diet and fluid restrictions, lack of physical exercise and the need for certain medications, phosphate binders in particular, by HD patients may contribute to constipation[27].

Similar to the results of previous studies[6-8,10,11], PD patients in our study experienced more pronounced reflux and eating dysfunction. These symptoms may be exacerbated by the filling of the abdominal cavity with dialysate fluid during PD, which increases the intra-abdominal pressure[28] and frequency of acid reflux episodes[29] as well as lowers the esophageal sphincter pressure. PD has also been reported as an independent pathophysiological factor for esophageal acid exposure[30]. Additionally, delayed gastric emptying in PD patients[13,31,32] and the glucose dialysate may play a metabolic role in gastric emptying[33]. An international cross-sectional study found that 33% of 224 CAPD patients were not well-nourished and 8% had severe malnutrition, of which one of the leading causes was eating dysfunction[34] that may have resulted from the associated food aversion, early satiety and changes in taste and smell[20]. Eating dysfunction could also be partly due to local or systemic circulatory insufficiency, hypergastrinemia and higher levels of ammonia and inflammation[35].

To our knowledge, this is the first study directly comparing the diversity of GI symptoms in HD and PD patients. GI symptoms were common in dialysis patients, but the prominent symptoms unexpectedly differed between patients undergoing different dialysis therapies. Although the majority of patients complained of few and minor symptoms, approximately 10% suffered from more than three GI symptoms, which occurred in a greater percentage of HD than PD patients. The underlying mechanism is not clear, but hemodynamic changes, delayed gastric emptying, loss of residual renal function, and inadequate dialysis, such as protein-bound uremic toxin that cannot be effectively cleared by HD, may all contribute.

In conclusion, the present study demonstrated a high prevalence of GI symptoms in HD and PD patients, with constipation, abdominal pain and diarrhea more frequent and severe in HD patients, and reflux more prominent in PD patients. However, the results are not in complete agreement with previous studies, thus necessitating further evaluation in a larger population of dialysis patients.

ACKNOWLEDGMENTS

We would like to thank Xie-Qin You and Xing Lu for their assistance with grammar and statistical analysis, respectively.

COMMENTS

Background

Gastrointestinal (GI) symptoms are commonly reported among patients with end-stage renal disease and correlate to nutritional status, life quality, and even mortality. Until now, little attention has been paid to these GI symptoms, especially in hemodialysis (HD) patients.

Research frontiers

The gastrointestinal symptom rating scale (GSRS) has been used widely for evaluating the presence of GI symptoms in the population. The authors in this study used the GSRS to evaluate more than two hundred dialysis patients, and compared the prevalence, severity and diversity of GI symptoms between patients receiving HD or peritoneal dialysis (PD).

Innovations and breakthroughs

Previous studies have presented cross-sectional descriptions of GI symptoms in PD and HD patients. The prevalence, severity and diversity of GI symptoms are directly compared in more than two hundred PD and HD patients in the present study. The results show that HD patients have a higher prevalence and severity of constipation, abdominal pain and diarrhea, whereas PD patients have a higher prevalence and severity of reflux and more severe eating dysfunction. Moreover, a greater number of HD patients presented with more than three GI symptoms.

Applications

The results of this study suggest that GI symptoms differ in patients with PD and HD, and clinicians should therefore have differential focus and treatments for patients with GI symptoms undergoing dialysis.

Terminology

Kt/V urea is an index used to quantify HD and PD treatment adequacy: *K* refers to the dialyzer clearance of urea, *t* is the dialysis time, and *V* is the volume of distribution of urea, approximately equal to the patient's total body water. The US National Kidney Foundation designates the *Kt/V* target as ⩾ 1.2 for HD and ⩾ 1.7/wk for PD patients.

Peer review

This is a good research study in which authors compare the GI symptoms in patients undergoing PD and HD. The results are interesting and suggest that GI symptoms differ between PD and HD patients, and clinicians should therefore evaluate and differentially treat GI symptoms in dialysis patients.

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P- Reviewer: Shu KH, Zouiten-Mekki L **S- Editor**: Nan J
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DOI: 10.3748/wjg.v20.i32.11376

World J Gastroenterol 2014 August 28; 20(32): 11376-11383
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

RETROSPECTIVE STUDY

Laparoscopic spleen-preserving splenic hilar lymphadenectomy in 108 consecutive patients with upper gastric cancer

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Supported by the National Key Clinical Specialty Discipline Construction Program of China, No. [2012]649

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Received: January 8, 2014 Revised: April 12, 2014
Accepted: July 24, 2014
Published online: August 28, 2014

Abstract

AIM: To evaluate the feasibility and short-term efficacy of laparoscopic spleen-preserving splenic hilar (No. 10) lymphadenectomy to treat advanced upper gastric cancer (AUGC).

METHODS: Between January and December 2012, 108 laparoscopic spleen-preserving No. 10 lymphadenectomy along with total gastrectomy with routine D2 lymphadenectomy were performed consecutively at our hospital to treat clinical T2-3 (cT2-3) upper gastric cancers. The preoperative clinical T stage was cT2 in 36 patients and cT3 in 72 patients. A prospectively designed database tracked the 108 patients, including the completeness of their medical records and the adequacy of follow-up. Patient clinicopathological characteristics, intraoperative and postoperative surgical outcomes, morbidity and mortality, lymph node (LN) dissection, and postoperative follow-up were analysed retrospectively.

RESULTS: Laparoscopic spleen-preserving No. 10 lymphadenectomy was successful in all 108 patients. The mean operation time was 169.3 ± 27.1 min, and the mean No. 10 lymphadenectomy time was 20.0 ± 5.7 min. The mean total blood loss was 46.2 ± 11.3 mL, and the mean blood loss from No. 10 lymphadenectomy was 14.3 ± 3.8 mL. The mean postoperative hospital stay was 11.9 ± 6.0 d. The intraoperative and postoperative morbidity rates were 3.7% and 12.0%, respectively; however, there was no postoperative mortality. A mean of 44.4 ± 17.6 LNs were retrieved from each specimen, including 3.0 ± 2.4 No. 10 LNs. Three patients (2.8%) with cT3 cancer had LN metastasis of the splenic hilus, including two patients with pathological T3 (pT3) and one patient with pathological T4a (pT4a) tumours, all located in the greater curvature. No splenic hilar LNs metastasis was evident in the patients with pT1 and pT2 tumours. At a median follow-up time of 18 mo (range, 12 to 23 mo), all patients were alive and none had experienced recurrent or metastatic disease.

CONCLUSION: Laparoscopic spleen-preserving No. 10 lymphadenectomy is feasible and effective to treat AUGC. Routine No. 10 lymphadenectomy may be unnecessary for AUGC without serosa invasion, unless T3 tumours are located in the greater curvature.

Key words: Stomach neoplasms; Spleen-preservation; laparoscopy; Gastrectomy; Lymphadenectomy

Core tip: Several studies have shown that laparoscopic spleen-preserving No. 10 lymphadenectomy is feasible for patients with upper gastric cancer; however, the sample sizes in these studies were small. Thus, the value of the procedure must be further evaluated with large sample studies, and it is debatable whether routine No. 10 lymphadenectomy should be performed for advanced upper gastric cancer (AUGC) without serosa invasion. Therefore, we evaluated the feasibility and short-term efficacy of laparoscopic spleen-preserving No. 10 lymphadenectomy in 108 consecutive patients with AUGC (cT2-3). In addition, early follow-up results were also presented.

Li P, Huang CM, Zheng CH, Xie JW, Wang JB, Lin JX, Lu J, Wang Y, Chen QY. Laparoscopic spleen-preserving splenic hilar lymphadenectomy in 108 consecutive patients with upper gastric cancer. *World J Gastroenterol* 2014; 20(32): 11376-11383 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11376.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11376

INTRODUCTION

Splenic hilar lymph nodes (No. 10 LNs) are LNs that are located in the splenic hilum, including those LNs adjacent to the splenic artery and distal to the pancreatic tail, on the roots of the short gastric arteries, and along the left gastroepiploic artery proximal to the first gastric branch[1]. Standard D2 LN dissection during total gastrectomy for advanced upper gastric cancer (AUGC) requires the removal of the No. 10 LNs[1]. Spleen-preserving No. 10 lymphadenectomy is technically feasible and safe for patients undergoing open surgery for upper gastric cancer[2], with lower postoperative morbidity and mortality rates than splenectomy[3-7]. Moreover, the radical effects and long-term survival rates were similar to those in patients who underwent splenectomy[8-12]. Total gastrectomy with spleen-preserving No. 10 lymphadenectomy is, therefore, increasingly used to treat patients with upper gastric cancer. Spleen-preserving D2 lymphadenectomy, however, requires an anatomical No. 10 lymphadenectomy, a procedure that is technically difficult because of the presence of intricate and complex vessels, and a narrow and deep space at the splenic hilum. Moreover, complete removal of No. 10 LNs is particularly difficult in obese patients and patients with splenic adhesions.

Laparoscopic gastrectomy with D2 lymphadenectomy is safe and feasible for patients with advanced gastric cancer[13-16]. Although several studies have shown that laparoscopic spleen-preserving No. 10 lymphadenectomy is feasible during total gastrectomy with D2 LN dissection for small numbers of patients with upper gastric cancer[17-19], few large-scale studies have assessed its success in patients with AUGC. Furthermore, it is debatable whether routine No. 10 lymphadenectomy should be performed during total gastrectomy for AUGC without serosa invasion. Therefore, in the current study, we evaluated the feasibility and short-term efficacy of laparoscopic spleen-preserving No. 10 lymphadenectomy in 108 consecutive patients with cT2-3 AUGC. In addition, early follow-up results were also presented.

MATERIALS AND METHODS

Patients

Between January and December 2012, 108 consecutive patients with cT2-3 AUGC underwent laparoscopic spleen-preserving No. 10 lymphadenectomy, along with total gastrectomy and routine D2 lymphadenectomy in the Department of Gastric Surgery, Fujian Medical University Union Hospital. Beginning in May 2007, the surgeon (Huang CM) in this study had performed more than 500 laparoscopy-assisted gastrectomies with D2 LN dissection in gastric cancer patients before attempting this procedure. Since then, a prospectively designed database has tracked all laparoscopy-assisted gastrectomies for gastric cancer. Moreover, the surgeon performed laparoscopic spleen-preserving No. 10 lymphadenectomy for advanced proximal gastric cancer using a left-sided approach[20]. This method was mastered after a learning curve of 40 patients[21]. The group of 108 consecutive patients was used for our retrospective analysis because of the completeness of their medical records and the adequacy of their follow-up. Upper gastric cancer was diagnosed by analysis of endoscopic biopsy specimens. Preoperative imaging studies were routinely performed following endoscopic examination, computed tomography (CT) scanning, abdominal ultrasonography (US) and endoscopic US. CT scans and multi-slice spiral CT angiography (MSCTA) were performed to assess preoperatively the splenic vascular anatomy (Figures 1 and 2). Advanced (cT2-T3) upper gastric cancer diagnosed by preoperative CT scanning and endoscopic US were enrolled in this study. Patients with clinical T1 (cT1) or clinical T4 (cT4) tumours, distant metastasis, or preoperative enlargement or integration of LNs were excluded. No patients had received preoperative chemoradiation therapy. Each preoperative patient was informed of the surgical procedure, including its advantages and risks. All patients provided written informed consent for the procedure before surgery, as well as for the publication of this report and any accompanying images.

In the current study, the No. 10 lymphadenectomy began with the surgeon using an ultrasonic scalpel to separate and reveal the end of the splenic arteries within the retropancreatic space at the superior border of the pancreatic tail, to divide the last short gastric artery. The No. 10 lymphadenectomy time referred to the time of this procedure. The blood loss during surgery was measured by estimating the volume of blood in the suction container and weighing the gauze with blood. Dissected LNs were classified according to the 3rd English edition of the Japanese classification of gastric carcinoma[1]. The

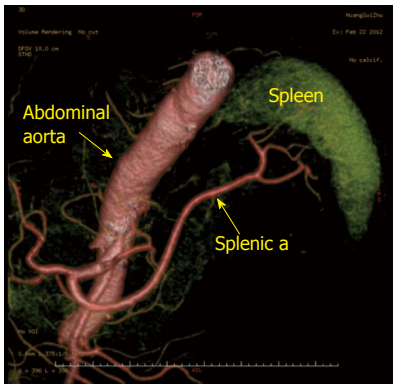

Figure 1 Preoperative computed tomography angiography showing the drainage of the splenic arteries. Abdominal aorta (arrow); splenic a (arrow). a: Artery.

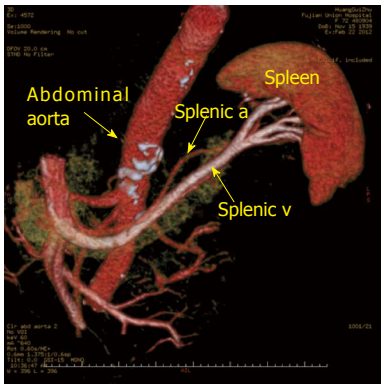

Figure 2 Preoperative computed tomography angiography showing the drainage of the splenic veins. Abdominal aorta (arrow); splenic a (arrow); splenic v (arrow). a: Artery; v: Vein.

clinical and pathological stagings were in accordance with the American Joint Committee on Cancer (AJCC) seventh edition of Gastric Cancer TNM Staging[22]. Follow-up was performed by trained investigators every 3 mo. Routine follow-up comprised a physical examination, laboratory tests, chest radiography, abdominopelvic ultrasonography or CT scans. Survival time was calculated from the time of surgical intervention until the last date of contact (December 31, 2013).

Surgical procedures

Patient positioning: The patient was placed in the reverse Trendelenburg position with the head elevated approximately 15° to 20°, and tilted with the left side up approximately 20° to 30°. A 10-mm trocar for the laparoscope was inserted below the umbilicus; a 12-mm trocar was inserted in the left upper quadrant as a major hand port; a 5-mm trocar was inserted in the left lower quadrant as an accessory port; a second 5-mm trocar for exposure was inserted in the left upper quadrant; and a third 5-mm trocar for exposure was inserted in the right lower quadrant. The surgeon stood on the left side of the patient; the assistant surgeon was on the right side; and the camera operator was situated between the patient's legs.

Other lymphadenectomy: The gastrocolic ligament was divided using an ultrasonic scalpel along the border of the transverse colon. The right gastroepiploic vein and the right gastroepiploic artery were vascularised and divided to dissect the No. 6 LNs. The stomach was lifted toward the head to expose the gastropancreatic fold. The LNs along the proximal splenic artery (No. 11p) at the upper border of pancreatic body were removed. The dissection was then continued rightward. The fatty connective tissue, including the LNs along the celiac trunk (No. 9), the left gastric artery (No. 7), and the common hepatic artery (No. 8a) were removed en-block with the left gastric vein and the left gastric artery being vascularised and divided. The LNs around the right gastric artery (No. 5) and along the surface of the proper hepatic artery (No. 12a) were then dissected and removed. Subsequently, the liver was held up to divide the hepatogastricum ligament along the lower border of the liver and the LNs around lesser curvature (No. 3) were removed. Finally, the phrenoesophageal membrane and both vagus nerves were divided and the LNs around the abdominal oesophagus (No. 1 and 2) were dissected.

No. 10 lymphadenectomy: The patient was subsequently tilted, with the left side up approximately 20° to 30° and subjected to a 20° upward head tilt. The surgeon then moved to stand between the patient's legs, and the assistant and camera operator were both on the patient's right side. Before surgery, the assistant placed the greater omentum behind the stomach to keep the visual field clear, and pulled and tensed the gastrosplenic ligament. The surgeon gently pressed the tail of the pancreas toward the lower left, exposing the splenic hilum. The surgeon separated the membrane of the body and tail of the pancreas to reach the posterior space at the superior border of the pancreas, and opened the vascular envelope at the end of the splenic arteries. The surgeon dissected away the lymphatic fatty tissue on the surface of the inferior splenic lobar artery from the lower pole of the spleen, vascularised the left gastroepiploic artery issuing from the inferior splenic lobar artery, and then cut the left gastroepiploic artery (No. 4sb) from the origin. The assistant then placed the free omentum between the liver and the stomach and continually pulled the posterior wall of the fundus and body of the stomach to the upper right. The surgeon gently pressed the pancreas to fully reveal the retropancreatic space and the space inside the splenorenal ligament. The surgeon then tracked the termini of the splenic vessels along the completely vascularised the lower lobar vessels of the spleen within the space inside the splenorenal ligament. Next, the surgeon carefully dissected the fatty lymphatic tissue around the splenic vessels (No. 11d) along the latent anatomic spaces on the surface of the splenic vessels. At this time, the assistant gently pulled up the lymphatic fatty tissue at the surface of the inferior splenic lobar artery. Starting from the root

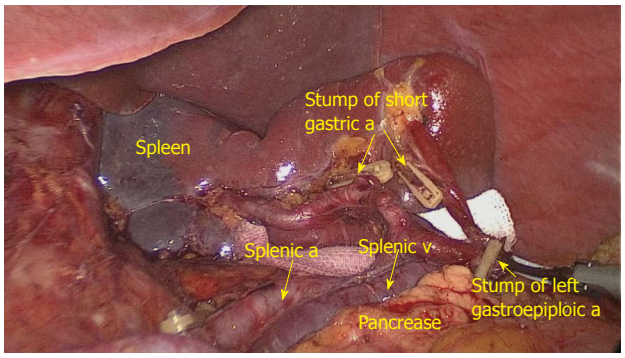

Figure 3 No. 10 lymph nodes lymphadenectomy at the front of the splenic vessels (anterior view). Dividing left gastroepiploic a (arrow); dividing short gastric a (arrow); splenic a (arrow); splenic v (arrow); a: Artery; v: Vein.

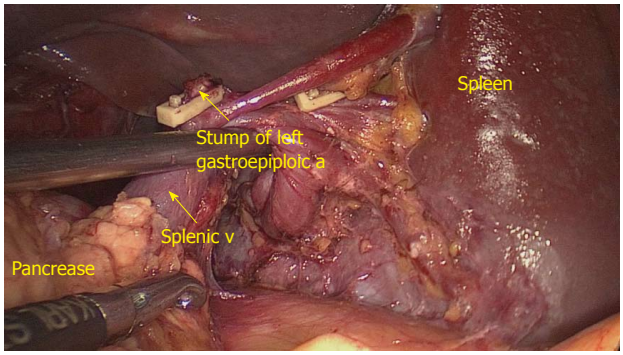

Figure 4 No. 10 lymph nodes lymphadenectomy behind the splenic vessels (posterior view). Dividing left gastroepiploic a (arrow); splenic vein (arrow); a: Artery; v: Vein.

of the left gastroepiploic artery, the surgeon, using the non-functional face of the ultrasonic scalpel, closed the surface of the inferior splenic lobar artery. The surgeon used the ultrasonic scalpel to carefully dissect the lymphatic fatty tissue and to vascularise the inferior splenic lobar artery. After the latter became visible, the short gastric arteries issuing from the inferior splenic lobar artery were skeletonised and divided at their roots, resulting in complete vascularisation of the inferior splenic lobar artery. The fatty tissues and gastric tissues were pulled up by the assistant, and the surgeon dissected the lymphatic fatty tissue on the surface of the superior splenic lobar artery, starting from the root of the artery towards the upper pole of the spleen, as described for vascularisation of the inferior splenic lobar artery. One branch of the short gastric artery issuing from the superior splenic lobar artery was skeletonised and divided at its root. This procedure resulted in LN dissections at the front of the splenic vessels (Figure 3). The assistant then pulled the root of the inferior splenic lobar artery towards the upper right, revealing the lymphatic fatty tissue behind the splenic hilum. The latter was pulled up by the surgeon towards the lower left to maintain tension. The lymphatic fatty tissue behind the splenic hilum was then dissected (No. 10) (Figure 4). A piece of gauze was placed behind the splenic hilum to indicate that the vessels had been vascularised and the LNs had been completely dissected.

Table 1 Baseline demographic and clinicopathological characteristics of patients

Characteristic		Value
Gender	Male/female	87/21
Age (yr)		62 ± 9
Tumour size (cm)		5.0 ± 2.6
BMI (kg/m^2)		22.1 (14.5-34.5)
Tumour location	Lesser curvature/greater curvature/anterior wall/posterior wall/circumferential involvement	30/21/15/19/23
Pathological type	Differentiated/undifferentiated type	43/65
cT stage	T2/T3	36/72
pT stage	T1/T2/T3/T4a	12/14/73/9
pN stage	N0/N1/N2/N3	31/17/23/37
TNM stage	ⅠA/ⅠB/ⅡA/ⅡB/ⅢA/ⅢB/ⅢC	9/8/19/14/23/29/6

Data are expressed as mean ± SD. BMI: Body mass index; cT stage: Clinical tumour stage; pT stage: Pathological tumour stage.

Digestive tract reconstruction: The duodenum was transected 2 cm below the pylorus with a 60-mm laparoscopic cartridge linear stapling device through the major hand port. Finally, a longitudinal laparotomy was performed using a 6-8 cm skin incision at the epigastrium, and the specimen was extracted from the peritoneal cavity. The transaction of the oesophagus and Roux-en-Y oesophagojejunostomy was carried out using a circular stapler. A side-to-side jejunojejunostomy was performed by hand suture.

RESULTS

Patient clinicopathological characteristics

The 108 patients included 87 males (80.6%) and 21 females (19.4%) with a mean age of 62.5 ± 9.2 years (range, 24-82 years) and mean body mass index (BMI) of 22.1 ± 2.9 kg/m^2 (range: 14.5-34.5 kg/m^2). The preoperative clinical T stage was cT2 in 36 patients (33.3%) and cT3 in 72 patients (66.7%). The postoperative pathological TNM stages included pT1 (n = 12), pT2 (n = 14), pT3 (n = 73), and pT4a (n = 9); pN0 (n = 31), pN1 (n = 17), pN2 (n = 23), and pN3 (n = 37); IA (n = 9), IB (n = 8), IIA (n = 19), IIB (n = 14), IIIA (n = 23), IIIB (n = 29) and IIIC (n = 6) (Table 1).

Intraoperative and postoperative surgical outcomes

For all 108 patients, the mean operation time was 169.3 ± 27.1 min, and the mean No. 10 lymphadenectomy time was 20.0 ± 5.7 min. The mean estimated blood loss was 46.2 ± 11.3 mL, and the mean estimated blood loss for No. 10 lymphadenectomy was 14.3 ± 3.8 mL. The mean times to first flatus, fluid diet, and soft diet were 3.4 ± 1.1, 4.7 ± 1.6 and 8.3 ± 4.2 d, respectively, and the mean postoperative hospital stay was 11.9 ± 6.0 d (Table 2).

Table 2 Intraoperative and postoperative surgical outcomes

Item	Value
Operation time (min)	169.3 ± 27.1
Blood loss (mL)	46.2 ± 11.3
No. 10 lymphadenectomy (min)	20.0 ± 5.7
No. 10 lymphadenectomy blood loss (mL)	14.3 ± 3.8
Time to first flatus (POD)	3.4 ± 1.1
Time to fluid diet (POD)	4.7 ± 1.6
Time to soft diet (POD)	8.3 ± 4.2
Hospital stay (POD)	11.9 ± 6.0

Data are expressed as mean ± SD. No. 10 lymphadenectomy: Splenic hilar lymphadenectomy; POD: Postoperative days.

Table 3 Intraoperative and postoperative complications

Item	Value	Incidence
Intraoperative complications (*n*)	4	3.7%
Transverse colon injury	1	
Spleen injury	1	
Left gastric vein bleeding	1	
Gastric short arteries bleeding	1	
Postoperative complications (*n*)	13	12.0%
Pulmonary infection	8	
Abdominal infection	2	
Anastomotic leakage	1	
Intestinal obstruction	1	
Chylous fistula	1	

Morbidity and mortality

Four patients experienced intraoperative complications, giving an intraoperative morbidity rate of 3.7%. One patient experienced each of the following complications: injury to the transverse colon, injury to the splenic envelope, bleeding from the gastric coronary vein and bleeding from the gastric short arteries. All complications were treated during successfully laparoscopic surgery. No patient required conversion to laparotomy, and no patient required splenectomy because of intraoperative injury to the splenic blood vessels or the spleen itself. Postoperative complications occurred in 13 patients, giving a morbidity rate of 12.0%. These complications included abdominal infection in two patients, pulmonary infection in eight patients, inflammatory intestinal obstruction in one patient, chylous fistula in one patient, and anastomotic leakage in one patient. These postoperative complications were all successfully treated with conservative methods, and none of these patients required a second operation (Table 3). No patient experienced an operative splenic infarction, haemorrhage of the splenic blood vessels, or complications of spleen itself. The 30-d mortality rate for the total patient population was 0%.

LN dissection

The total number of LNs in all 108 patients was 4797, with a mean of 44.4 ± 17.6 LNs retrieved from each specimen. The total number of No. 10 LNs in all patients was 327, with a mean of 3.0 ± 2.4 No. 10 LNs retrieved per patient. Three patients (2.8%) had LN metastasis of the splenic hilus, including two patients with pT3 tumours and one patient with pT4a tumours, all located in the greater curvature (Table 4). There was no No. 10 LN metastasis in the patients with pT1 and pT2 tumours.

Table 4 Lymph nodes dissection results

Item	Value
Total No. of retrieved LNs	4797
Mean No. of retrieved LNs	44.4 ± 17.6
Total No. of retrieved No. 10 LNs	327
Mean No. of retrieved No. 10 LNs	3.0 ± 2.4
Total No. of No. 10 LNs metastasis	3
No. of No. 10 LNs metastasis in pT3	2
No. of No. 10 LNs metastasis in pT4a	1
No. 10 LNs metastasis rate	2.8%
No. 10 LNs metastasis rate in pT3	2.7%
No. 10 LNs metastasis rate in pT4a	11.1%

Data are expressed as mean ± SD. LN: Lymph node; No. 10 LN: Splenic hilar lymph node; pT3: Pathological T3 stage; pT4a: Pathological T4a stage.

Postoperative follow-up

The 108 patients were followed up for a median 18 mo (range: 12-23 mo). No patient died or experienced tumour recurrence or metastasis during the follow-up period.

DISCUSSION

D2 lymphadenectomy, including the removal of No. 10 LNs, has become the standard surgical procedure for patients with curable AUGC[1,6]. In recent years, with advances in surgical concepts, improvements in the anatomical techniques and the progress of organ retention, spleen-preserving No. 10 LN dissection has been used increasingly for AUGC patients[2,7,9,12]. However, this procedure is technically difficult, not only because of the intricate and complex blood vessels, but also because of the deep and limited operative space in the splenic hilum. On the one hand, in open surgery, the complete removal the No. 10 LNs often requires the mobilisation of the spleen from the abdominal cavity, which obviously increases patient trauma, elongates operation time, and is especially difficult for obese patients and patients with splenic adhesions. On the other hand, maintaining the spleen within the abdominal cavity and performing spleen-preserving No. 10 LN dissection directly would not completely remove all LNs, because the exposure would be insufficient. Similar to open surgery, spleen-preserving No. 10 LN dissection is also one of the most difficult procedures in laparoscopic surgery. Previously, a few studies have reported the feasibility of laparoscopic spleen-preserving splenic hilar LN dissection for AUGC[17-19] patients; however, the sample sizes in these studies were small; thus, the value of the procedure needed to be further evaluated by studies with large samples. According to the 3rd English edition of Japanese classification of gastric carcinoma[1], splenic hilar lymphadenec-

tomy is unnecessary for cT1 tumours and laparoscopic surgery applied to cT4 tumours has been controversial. In the current study, therefore, we studied the feasibility and short-term efficacy of laparoscopic spleen-preserving No. 10 lymphadenectomy in 108 consecutive patients with stage cT2-T3 upper gastric cancer. Our data showed that the average time needed for No. 10 LN dissection was approximately 20 min, with less bleeding and shorter postoperative hospital stays, suggesting that laparoscopic spleen-preserving No. 10 lymphadenectomy is technically feasible.

Previous studies reported that the intraoperative complication rate of laparoscopic gastric surgery was 2.6%-4.4%[23,24]. Consistent with these findings, we observed intraoperative complications in four of 108 patients (3.7%). None of our patients required conversion to laparotomy, and no patient required splenectomy because of injury to the spleen or splenic blood vessels. Postoperative complications were reported in 8.7%-25.0% of patients who underwent open spleen-preserving No. 10 lymphadenectomy for upper gastric cancer[2,9,11,12], and a recent study reported postoperative complications in two of 15 (13.3%) patients with upper gastric cancer who underwent laparoscopic spleen-preserving No. 10 lymphadenectomy[17]. In the current study, we found that 13 of 108 patients (12.0%) experienced postoperative complications, but no patient died within 30-d of follow-up, suggesting that laparoscopic spleen-preserving No. 10 LNs dissection is safe and does not increase postoperative morbidity and mortality rates. In our experience, the keys to successful No. 10 LN dissection are a skilled laparoscopic technique, familiarity with the minimally invasive vascular anatomy of the splenic hilum area, and a cooperative surgical team. Moreover, the laparoscope, with its unique perspective, lighting and amplification, can more clearly visualise the splenic vasculature, nerves, fascia and other structures, thereby reducing damage to the splenic vessels and spleen, and assisting the surgeon in performing spleen-preserving No. 10 lymphadenectomy without splenic mobilisation.

The number of dissected LNs is an important assessment of the outcome of LN dissection. The average number of No. 10 LNs dissected per patient has been reported as three LNs during open radical surgery for upper gastric cancer involving splenectomy[25] and 1.7 LNs during open radical surgery with spleen-preserving No. 10 lymphadenectomy[26]. During laparoscopic spleen-preserving No. 10 lymphadenectomy, the average numbers of No. 10 LNs dissected per patient were 2.7[17] and 2.6[19], indicating that a similar number of No. 10 LNs were dissected during laparoscopic and open surgery. In the current study, the average number of No. 10 LNs dissected was 3.0, which was similar to the other reports. No. 10 LNs are prone to metastasis in AUGC[25], and the metastasis rate to the No. 10 LNs reportedly ranges from 5.1% to 20.9%[9-11,27,28]. Moreover, the No. 10 LNs metastasis rate is related to the tumour location, depth of invasion, other total LNs metastasis status and size of the primary tumour[9-11,27,28]. In the current study, we observed metastases in these LNs in only three of 108 patients (2.8%), including two patients with pT3 and one patient with pT4a tumours, all located in the greater curvature; however, there was no No. 10 LNs metastasis in patients with pT1 and pT2 tumours. Therefore, this present study suggested that routine No. 10 lymphadenectomy may be unnecessary for AUGC without serosa invasion, unless T3 tumours are located in the greater curvature.

Patient survival after radical gastrectomy is important to evaluate its efficacy. The short and long term survival rates were greater in patients undergoing open spleen-preserving No. 10 lymphadenectomy for the treatment of upper stomach cancer[4-6]. In Hyung's study, none of the 15 patients who underwent a laparoscopic procedure died or experienced tumour recurrence after a median follow-up period of 21 mo[17]. We found that after a median follow-up time of 18 mo, none of our patients experienced recurrence or metastasis. Longer follow-up periods, however, are required to determine the long-term efficacy of this procedure.

In conclusion, laparoscopic spleen-preserving No. 10 lymphadenectomy is feasible and effective for patients with AUGC. However, routine No. 10 lymphadenectomy may be unnecessary for AUGC without serosa invasion, unless T3 tumours are located in the greater curvature. In addition, multi-centre, prospective, randomised controlled studies involving greater numbers of patients and longer follow-up times, are needed to confirm its long-term efficacy.

COMMENTS

Background

Standard D2 lymph node (LN) dissection during total gastrectomy for advanced upper gastric cancer (AUGC) requires the removal of the No. 10 LNs, according to the 3rd English edition of Japanese classification of gastric carcinoma. Total gastrectomy with spleen-preserving No. 10 lymphadenectomy is increasingly used in open surgery to treat patients with upper gastric cancer. Several studies have shown that laparoscopic spleen-preserving No. 10 lymphadenectomy is feasible for patients with upper gastric cancer; however, the sample sizes in these studies were small, and the value of the procedure must be further evaluated by studies with large sample sizes. Furthermore, it remains controversial whether routine No. 10 lymphadenectomy should be performed for AUGC without serosa invasion.

Research frontiers

Laparoscopic spleen-preserving No. 10 lymphadenectomy has been difficult to accomplish because of the possibilities of injury to splenic vessels and the parenchyma of the spleen or pancreas.

Innovations and breakthroughs

The results of the current study demonstrate that the average time needed for laparoscopic spleen-preserving No. 10 lymphadenectomy was approximately 20 min, and included less bleeding and shorter postoperative hospital stays. Moreover, the intraoperative and postoperative morbidity rates were 3.7% and 12.0%, respectively, and there was no postoperative mortality. At the same time, a mean of 3.0 ± 2.4 No. 10 LNs were retrieved per patient. Three patients (2.8%) had LN metastasis of the splenic hilus, including two patients with pT3 and one patient with pT4a tumours, all located in the greater curvature. At a median follow-up of 18 mo (range, 12 to 23 mo), no patient died or experienced tumour recurrence or metastasis during the follow-up period.

Applications

The study results suggest that laparoscopic spleen-preserving No. 10 lymphad-

enectomy is feasible and effective for AUGC. Routine No. 10 lymphadenectomy may be unnecessary for AUGC without serosa invasion, unless T3 tumours are located in the greater curvature. The results should encourage more surgeons to perform laparoscopic total gastrectomy with No. 10 lymphadenectomy and will aid the acceptance of this procedure as a surgical option for AUGC patients.

Terminology

For spleen-preserving No. 10 lymphadenectomy, surgeons do not need to remove the spleen during the No. 10 lymphadenectomy when performing total gastrectomy with D2 LN dissection. Body mass index was used as an objective index to indicate massive obesity. The cut-off value was chosen according to the World Health Organisation guidelines for the Western Pacific region.

Peer review

This is a good work in which the authors evaluate the feasibility and short-term efficacy of laparoscopic spleen-preserving No. 10 lymphadenectomy for AUGC. Congratulations to the authors for the excellence of their work. All the contents in this study are appropriately presented. This manuscript is well written and documented. Additionally, this manuscript adds new knowledge to the literature.

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P- Reviewer: Abd Ellatif ME, Coskun A, Ferreira Caboclo JL
S- Editor: Ma N **L- Editor**: Stewart G **E- Editor**: Zhang DN

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DOI: 10.3748/wjg.v20.i32.11384

World J Gastroenterol 2014 August 28; 20(32): 11384-11393
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

OBSERVATIONAL STUDY

Adjuvant heparanase inhibitor PI-88 therapy for hepatocellular carcinoma recurrence

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Supported by NIH Clinical Trial Registration, No. NCT00247728 (this trial was cosponsored by Progen Industries Limited, Brisbane, Australia and Medigen Biotechnology Corporation, Taipei, Taiwan) to Chen PJ, Lai KL and Chang SSC; Taiwan Liver Disease Consortium, the National Research Program for Biopharmaceuticals, and the National Science Council, Taiwan, NSC100-2325-B-002-052; NSC102-2325-B-002-079
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Received: November 16, 2013 Revised: February 12, 2014
Accepted: May 12, 2014
Published online: August 28, 2014

Abstract

AIM: To demonstrate that administering heparanase inhibitor PI-88 at 160 mg/d is safe and promising in reducing hepatocellular carcinoma (HCC) recurrence for up to 3 year following curative resection.

METHODS: A total of 143 patients (83.1% of the 172 participants in the phase Ⅱ study) participated in the follow-up study. Of these patients, 50 had received no treatment, 48 had received 160 mg/d PI-88, and 45 had received 250 mg/d PI-88 during the phase Ⅱ trial. Safety parameters and the following efficacy endpoints were investigated: (1) time to recurrence; (2) disease-free survival; and (3) overall survival.

RESULTS: PI-88 at 160 mg/d delayed the onset and frequency of HCC recurrence, and provided a clinically significant survival advantage for up to 3 years after treatment compared with those of the control group: (1) the recurrence-free rate increased from 50% to 63%, and (2) time to recurrence at the 36th percentile was postponed by 78%. The efficacy of administering PI-88 at 250 mg/d was confounded by a high dropout rate (11 out of 54 patients). Additionally, subgroup analyses of patients with (1) multiple tumors or a single tumor ⩾ 2 cm; and (2) hepatitis B or C revealed that administering PI-88 at 160 mg/d conferred the most significant survival advantage (56.8% improvement in disease-free survival, P = 0.045) for patients with both risk factors for recurrence.

CONCLUSION: Administering PI-88 at 160 mg/d is a safe and well-tolerated dosage that may confer significant clinical benefits for patients with HCC.

Key words: Antiangiogenesis; Antimetastasis; Adjuvant

therapy; Disease-free survival; Heparanase inhibitor; Hepatocellular carcinoma; PI-88; Tumor recurrence

Core tip: A phase Ⅱ clinical trial demonstrated that heparanase inhibitor PI-88 at 160 mg/d is safe and promising in reducing hepatocellular carcinoma (HCC) recurrence for up to one year following curative resection. This observational follow-up study extended the follow-up period to 3 years. A total of 143 patients participated in the study. PI-88 at 160 mg/d delayed the onset and frequency of HCC recurrence, and provided a clinically significant survival advantage for up to 3 years after treatment. Subgroup analyses revealed that administering PI-88 at 160 mg/d conferred the most significant survival advantage for patients at high risk of recurrence.

Liu CJ, Chang J, Lee PH, Lin DY, Wu CC, Jeng LB, Lin YJ, Mok KT, Lee WC, Yeh HZ, Ho MC, Yang SS, Yang MD, Yu MC, Hu RH, Peng CY, Lai KL, Chang SSC, Chen PJ. Adjuvant heparanase inhibitor PI-88 therapy for hepatocellular carcinoma recurrence. *World J Gastroenterol* 2014; 20(32): 11384-11393 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11384.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11384

INTRODUCTION

Hepatocellular carcinoma (HCC) is currently the fifth most common cancer and the third leading cause of cancer related deaths worldwide[1]. Traditionally, HCC has been more prevalent in Asia because of the prevalence of hepatitis B virus (HBV) infection. However, the incidence of HCC in the United States and Europe has risen in recent years because of increases in the number of hepatitis C virus (HCV) infections, consequently generating more interest in HCC research and treatment worldwide[2].

Surgical resection is a potentially curative therapy used to treat early-stage HCC; however, 50% to 80% of resection patients experience recurrence within 5 years[3,4]. Although numerous treatments, including oral and regional chemotherapy, interferon α and β, preoperative chemoembolization, and adoptive immunotherapy, have been investigated to reduce HCC recurrence, inconsistent and inconclusive results have prevented the adoption of these treatments in clinical practice[5-7]. Hence, there remains a dire clinical need for an adjuvant therapy to reduce the risk of postresection HCC recurrence[4,7,8].

There are 2 main types of postresection HCC recurrence. Intrahepatic metastatic recurrences develop from undetectable HCC dissemination prior to resection. *De novo* recurrences develop multicentrically and metachronously in the background liver, usually in patients with cirrhosis or chronic hepatitis[4,8]. Intrahepatic metastatic recurrence typically occurs within 2 years following resection, and *de novo* recurrence typically occurs 2 years following resection[6]. Although researchers who have conducted relevant molecular studies can differentiate between these types of recurrences to determine appropriate treatment strategies, these strategies are not widely used in clinical settings[4,8]. Currently, for convenience, recurrence in clinical settings is categorized as early or late, occurring within or after 2 years postresection, to approximate the likely mode of recurrence. Ideally, adjuvant therapies used to decrease postresection recurrence can inhibit both types of recurrence[4].

PI-88, a heparanase inhibitor, reduces HCC recurrence through 3 mechanisms. By inhibiting heparin sulfate (HS) degradation, PI-88 (1) preserves the integrity of the extracellular matrix (ECM) and (2) suppresses the release of angiogenic and fibroblastic growth factors (GFs) from the ECM. Moreover, the strong affinity of PI-88 to GFs enables PI-88 to (3) aggregate released GFs and block their activity. The antiangiogenic property of PI-88 stems from its ability to antagonize GF reception, and thereby restrict the necessary blood supply for both intrahepatic metastatic and *de novo* tumor proliferation. The antimetastatic property of PI-88 may stem from its ability to preserve ECM integrity, and thereby decrease basement invasion to further suppress intrahepatic metastatic recurrences[9-12]. Considering its ability to perform these dual functions, PI-88 can potentially suppress both types of HCC recurrences.

To investigate PI-88 as an adjuvant therapy for HCC recurrence, a randomized, multicenter Simon's 2-stage design phase Ⅱ trial was previously conducted to determine its safety, optimal dosage, and preliminary efficacy. The study results indicated that administering 160 mg/d for 36 wk postresection was a safe and optimal dosage that increased recurrence-free survival at 48 wk[13]. This observational follow-up study to the phase Ⅱ trial was conducted to determine whether these effects lasted longer than 48 wk and improved overall survival.

In the previous phase Ⅱ study, the primary endpoint was the recurrence-free survival rate; in this follow-up study, its more conventional equivalent, disease-free survival (DFS), was assessed. The efficacy endpoints analyzed in this follow-up study included (1) time-to-recurrence (TTR); (2) DFS; and (3) overall survival (OS). DFS and OS were assessed because both are reliable, clinically relevant endpoints[8]. This follow-up study also included subgroup analyses and further investigation to prepare for the design of a double-blind, randomized phase Ⅲ confirmatory study.

MATERIALS AND METHODS

Summary of phase Ⅱ study methods

From June 2004 to December 2006, a randomized phase Ⅱ trial designed according to Simon's 2-stage design was conducted in 6 medical centers in Taiwan, to determine the safety and optimal dosage of PI-88 in the adjuvant setting[14]. In total, 215 patients were screened, and 172 patients were randomized into 3 groups: 58

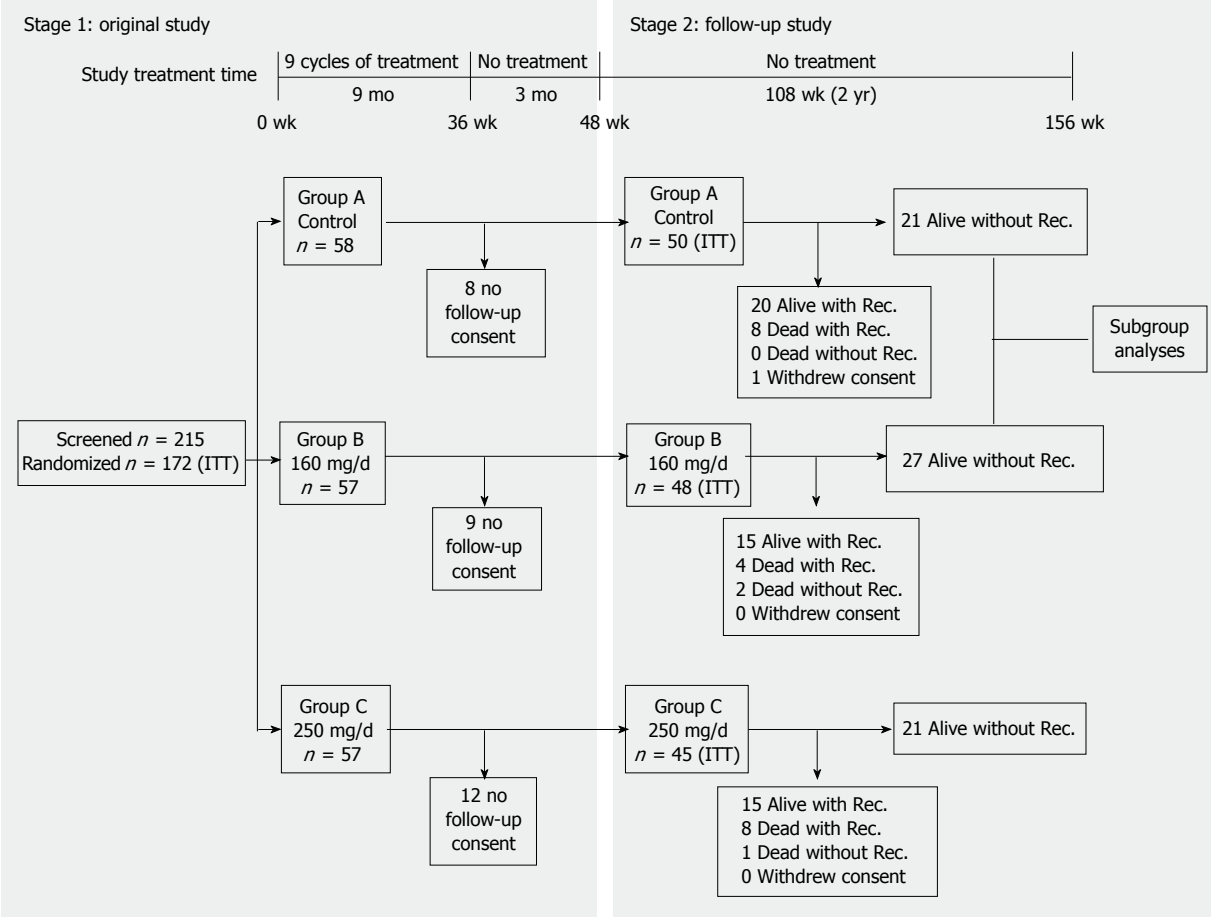

Figure 1 Graphic representation of phase Ⅱ and follow-up study design, timeline and cohort relationships. ITT: Intent-to-treat; Rec.: Recovery.

patients received no treatment, 57 patients received 160 mg/d PI-88, and 57 patients received 250 mg/d PI-88. PI-88 was provided in lyophilized powder form and produced by Progen Pharmaceuticals Limited (Brisbane, Queensland, Australia). As indicated in Figure 1, patients in Groups B and C received 9 cycles of the respective PI-88 treatments. Each cycle lasted 4 wk: weeks 1 through 3 each consisted of 4 consecutive days of treatment followed by 3 d without treatment; week 4 was a no-treatment week. After 9 treatment cycles (36 wk), no treatment was administered in the subsequent 12 wk before the final assessment in week 48. At the end of the study, data for assessing the safety and efficacy endpoints, including the tumor recurrence-free rate, TTR, and 1-year survival rate, were collected[13].

Follow-up study design

During the observational follow-up study, recurrence and survival data were collected for 2 years to examine the long-term efficacy of PI-88. All of the participants in the phase Ⅱ study were invited to participate in the follow-up study. In the follow-up study, the period between week 48, when the first patient in the phase Ⅱ trial completed his or her last visit during that week, and week 156, when the last patient completed his or her last 2-year follow-up visit, in January 2009 was investigated (Figure 1).

In both the clinical trial and the follow-up study, all of the patients received follow-up care according to Taiwanese standard-of-care guidelines: vital signs were checked, alfa fetoprotein and liver enzyme tests were conducted, and abdominal ultrasonograph and abdominal computed tomography (CT) scans were taken at outpatient clinics every 3 mo. CT scans and ultrasonographs were alternately performed every 1.5 mo. Additional CT scans were performed when HCC recurrence was suspected. In patients with HCC recurrence, treatment strategy was determined by the responsible investigator, basing on practice guidelines of individual institute. Treatment-related adverse events that occurred in the phase Ⅱ trial were also monitored throughout the follow-up study.

Ethical considerations

The follow-up protocol was approved by the institutional review board at each center and conformed to the ethical guidelines of the Declaration of Helsinki and local laws. Written informed consent was provided by the patient directly or by family members of patients who had passed away prior to the start of the follow-up study.

Patient demographics and baseline condition

As shown in Figure 1, 143 patients, or 83.1% of those in the phase Ⅱ study, participated in the follow-up study.

Table 1 Intent-to-treat patient demographics and baseline characteristic in the follow-up study *n* (%)

	Group A untreated (*n* = 50)[1]	Group B 160 mg/d (*n* = 48)	Group C 250 mg/d (*n* = 45)	Overall (*n* = 143)	*P*-value[2]
Age (yr)					
mean ± SD	55.9 ± 12.2	52.3 ± 12.6	54.3 ± 11.9	54.2 ± 12.2	0.3565
Age group (yr)					
Age < 65	36 (72.0)	38 (79.2)	37 (82.2)	111 (77.6)	0.4667
Age ≥ 65	14 (28.0)	10 (20.8)	8 (17.8)	32 (22.4)	
Sex					
Female	13 (26.0)	10 (20.8)	9 (20.0)	32 (22.4)	0.7445
Male	37 (74.0)	38 (79.2)	36 (80.0)	111 (77.6)	
Alcohol use					
Never or rarely	43 (86)	35 (72.9)	36 (80.0)	114 (79.7)	0.7547
Monthly	1 (2.0)	1 (2.1)	1 (2.2)	3 (2.1)	
Weekly	2 (4.0)	6 (12.5)	3 (6.7)	11 (7.7)	
Daily	4 (8.0)	6 (12.5)	5 (11.1)	15 (10.5)	
Clip stage					
0	30 (60.0)	25 (52.1)	26 (57.8)	81 (56.6)	0.6169
1	13 (26.0)	12 (25.0)	10 (22.2)	35 (24.5)	
2	4 (8.0)	6 (12.5)	7 (15.6)	17 (11.9)	
3	1 (2.0)	5 (10.4)	2 (4.4)	8 (5.6)	
4	2 (4.0)	0	0	2 (1.4)	
Ecog performance status score					
0	42 (84.0)	40 (83.3)	40 (88.9)	122 (85.3)	0.6496
1	7 (14.0)	8 (16.7)	5 (11.1)	20 (14.0)	
2	1 (2.0)	0	0	1 (0.7)	
Child-Pugh score					
5/6	48 (96.0)	46 (95.8)	43 (95.6)	137 (95.8)	0.6702
7	1 (2.0)	2 (4.2)	2 (4.4)	5 (3.5)	
8	1 (2.0)	0	0	1 (0.7)	
New York Heart Association classification of functional capacity class activity					
Class I	48 (96.0)	47 (97.9)	44 (97.8)	139 (97.2)	0.8144
Class II	2 (4.0)	1 (2.1)	1 (2.2)	4 (2.8)	
Differentiation of tumor					
Well differentiated	2 (4.0)	7 (14.6)	4 (8.9)	13 (9.1)	0.2487
Moderately differentiated	34 (68.0)	23 (47.9)	26 (57.8)	83 (58.0)	
Poorly differentiated or anaplasia	14 (28.0)	18 (37.5)	15 (33.3)	47 (32.9)	
Liver cirrhosis					
Absence	19 (38.0)	20 (41.7)	12 (26.7)	51 (35.7)	0.6023
Presence	28 (56.0)	24 (50.0)	29 (64.4)	81 (56.6)	
Not assessed	3 (6.0)	4 (8.3)	4 (8.9)	11 (7.7)	
Hepatitis activity					
Absence	7 (14.0)	4 (8.3)	5 (11.1)	16 (11.2)	0.8234
Presence	34 (68.0)	35 (72.9)	29 (64.4)	98 (68.5)	
Not assessed	9 (18.0)	9 (18.8)	11 (24.4)	29 (20.3)	
Vein invasion (microscopic)					
Absence	42 (84.0)	36 (75.0)	36 (80.0)	114 (79.7)	0.7375
Presence	8 (16.0)	11 (22.9)	8 (17.8)	27 (18.9)	
Not assessed	0 (0)	1 (2.1)	1 (2.2)	2 (1.4)	
Macro vascular invasion					
Absence	47 (94.0)	42 (87.5)	42 (93.3)	131 (91.6)	0.4493
Presence	3 (6.0)	6 (12.5)	3 (6.7)	12 (8.4)	

[1]Includes 1 patient who withdrew consent during follow-up study; [2]*P*-value on Age is by using analyses of variance, on CLIP stage by using Cochran-Mantel-Haenszel modified ridit scores for mean scores difference, on others by using χ^2 test. Differences in patient demographics and baseline characteristics were not statistically significant.

Of these patients, 50 patients received no treatment (Group A), 48 patients received 160 mg/d PI-88 (Group B), and 45 patients received 250 mg/d PI-88 (Group C). A logistic regression model was used to explore disparities in the baseline characteristics (1) among Groups A, B, and C in the follow-up study; and (2) between the phase Ⅱ and follow-up study cohorts. The investigated baseline factors included tumor features, Cancer of the Liver Italian Program score, Eastern Cooperative Oncology Group performance score, Child-Pugh status, viral hepatitis activity, and vascular invasion (Table 1).

Statistical analysis

Of the participants in the phase Ⅱ trial, 24 patients did not participate in the follow-up study. These individuals are not represented in the follow-up study data; they were counted at the end of the phase Ⅱ study (week 48) to achieve conservative endpoint estimations for all of the

Table 2 Other treatment or medication for recurrent hepatocellular carcinoma during the 156 wk follow-up period *n* (%)

Anti-HCC therapy	Group A untreated (*n* = 58)	Group B 160 mg/d (*n* = 56)	Group C 250 mg/d (*n* = 54)	Total (*n* = 168)
At least one shown below	22 (37.9)	17 (30.4)	22 (40.7)	61 (36.3)
Chemotherapy	3 (5.2)	4 (7.1)	4 (7.4)	11 (6.5)
Percutaneous ethanol injection therapy	2 (3.4)	3 (5.4)	3 (5.6)	8 (4.8)
Radiofrequency ablation	2 (3.4)	3 (5.4)	3 (5.6)	8 (4.8)
Radiotherapy	3 (5.2)	1 (1.8)	1 (1.9)	5 (3.0)
Surgical resection	5 (8.6)	5 (8.9)	7 (13.0)	17 (10.1)
Transcatheter arterial chemoembolization	18 (31.0)	11 (19.6)	15 (27.8)	44 (26.2)
Thalidomide	2 (3.4)	1 (1.8)	1 (1.9)	4 (2.4)
Liver transplantation	0 (0.0)	1 (1.8)	1 (1.9)	2 (1.2)
Sorafenib	1 (1.7)	1 (1.8)	1 (1.9)	3 (1.8)
New clinical trial	3 (5.1)	1 (1.8)	6 (11.3)	10 (6.0)

HCC: Hepatocellular carcinoma.

3-year study data.

Efficacy endpoints of interest in this study included (1) TTR; (2) DFS; and (3) OS. TTR, DFS, and OS were respectively defined as the time until each of the following events occurred: recurrence only, recurrence or death (unrelated to HCC recurrence), and death only; patients who withdrew consent were included until their drop-out times. The DFS event time for patients whose deaths were recurrence-related was defined as the time of recurrence.

The Kaplan-Meier estimator was used to determine TTR, DFS, and OS probabilities. TTR Hazard ratios were calculated using a Log rank test [logS(t, treated)/logS(t, untreated)]. Fisher's exact test was used to determine the statistical significance of the differences in the TTR, DFS, and OS probabilities among the 3 groups.

Subgroup analyses

Subgroup analyses were conducted to determine the efficacy of PI-88 administered at 160 mg/d in reducing HCC recurrence in patients with tumors and host factors that influence recurrence. Patients with multiple tumors, or a single tumor ⩾ 2 cm, were included in the intermediate-risk subgroup. Patients with both (1) multiple tumors or a single tumor ⩾ 2 cm; and (2) chronic hepatitis B or C infection were included in the high-risk subgroup[5]. DFS, the rate of DFS improvement, and statistical significance were calculated for both of the subgroups with respect to the controls.

To investigate the effects of patient population heterogeneity as suggested by Forner and Roayaie[15], statistical analyses comparing the recurrence trends between cohorts, including and excluding Child-Pugh class B patients, were conducted.

RESULTS

Summary of phase Ⅱ study results

In addition to being safe and well-tolerated throughout the 9 treatment cycles, PI-88 administered at 160 mg/d demonstrated the following efficacy improvements compared with those of the control group: (1) the recurrence-free rate increased from 50% to 63% and (2) TTR at the 36th percentile was postponed by 78%. The efficacy of administering PI-88 at 250 mg/d was confounded by a high dropout rate (11 out of 54 patients); the higher dosage was not determined to confer additional clinical advantages. Overall, the results supported the possible efficacy of administering PI-88 at 160 mg/d in decreasing and delaying recurrence, and prolonging disease-free survival in the short term[13,15].

Patient demographics

Statistical analyses revealed that the patient baseline demographics and tumor statuses among the 3 groups in the follow-up cohort, and between the respective follow-up and phase Ⅱ study groups, were not significant.

Treatment of recurrent HCC

Overall, during the 156 wk follow-up period, 61 (36.3%) of the 168 patients received treatment for recurrent HCC. The details of treatment strategies among different groups of patients are shown in Table 2.

Safety profiles

As shown in Table 3, only thrombocytopenia and elevated serum aminotransferase levels persisted from the phase Ⅱ study to the follow-up study. Common treatment-related adverse effects (AEs), such as neutropenia, injection site pain and hemorrhage, and the prolongation of activated partial thromboplastin time, were not observed in the follow-up study up to week 156[13]. Higher incidences of elevated serum aminotransferase levels in the 250 mg/d group were detected before week 60 (3 mo of follow-up). However, in the absence of antiHBV or anti-HCV treatment, most liver enzyme abnormalities returned to normal by the end of the first year of the follow-up study (week 102).

Compliance: We defined compliance in those subjects who received ⩾ 80% of the required doses (12 doses/cycle × 9 cycles × 80%). Rate of compliance in Groups B and C is shown in Table 4, categorized by drop-out status. In general, compliance was lower in Group C than that in Group B. For those subjects remaining in the phase Ⅱ study, the rate of compliance was also lower in

Table 3 Adverse events (with > 5% incidence) possibly related to treatment observed at the end of the phase Ⅱ study and in the follow-up study *n* (%)

Timeline	Week-48 End of phase Ⅱ study	Week-60 3 mo into follow-up study	Week-102 1 yr into follow-up study	Week-156 End of follow-up study
MedDRA system				
Blood and lymphatic system disorders: Thrombocytopenia				
Group B: 160 mg/d	2 (4.2)	2 (4.2)	2 (4.2)	0 (0.0)
Group C: 250 mg/d	3 (6.7)	3 (6.7)	3 (6.7)	3 (6.7)
P-value[1]	0.671	0.671	0.671	0.109
Investigations; elevated ALT/elevated AST				
Group B: 160 mg/d	2 (4.2)/3 (6.3)	2 (4.2)/3 (6.3)	2 (4.2)/1 (2.1)	1 (2.1)/0 (0.0)
Group C: 250 mg/d	7 (15.6)/7 (15.6)	7 (15.6)/7 (15.6)	2 (4.4)/1 (2.2)	1 (2.2)/1 (2.2)
P-value[1]	0.0843/0.1893	0.0843/0.1893	1.0000/1.0000	1.0000/0.4839

[1]Fisher's exact test. Treatment-related adverse events that were observed only in the phase Ⅱ study are excluded. All adverse events in the 160 mg/d treatment group reverted to baseline levels by the end of the follow-up study. Adverse events were more frequently observed in the 250 mg/d treatment group. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

Table 4 Rate of compliance[1] categoried by drop-out status *n* (%)

Drop-out status	Group B 160 mg/d (*n* = 56)	Group C 250 mg/d (*n* = 54)	*P*-value[2]
Drop-outs without recurrence	5	11	0.214
< 80% compliance	3 (60.0)	10 (90.9)	
⩾ 80% compliance	2 (40.0)	1 (9.1)	
Non-withdrawal subjects	51	43	0.171
< 80% compliance	11 (21.6)	15 (34.9)	
⩾ 80% compliance	40 (78.4)	28 (65.1)	

[1]⩾ 80% compliance denotes received ⩾ 80% of required doses (12 doses/cycle × 9 cycles × 80); [2]Fisher's exact test.

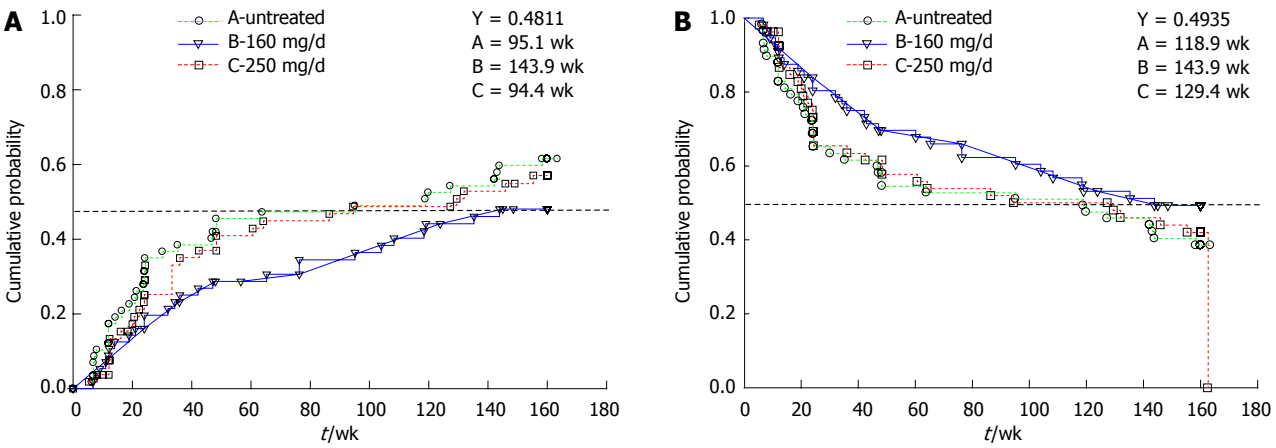

Figure 2 3-year probability for Groups A, B and C. A: Time-to-recurrence; B: Disease-free survival.

Group C compared with Group B although not statistically significant.

Efficacy endpoint analyses

TTR: TTR at the 48th percentile for Groups A, B, and C occurred in weeks 95.1, 143.9, and 94.4, respectively (Figure 2A). Compared with Group A, the TTR of Group B increased by 51.3%, which represents a hazard ratio of 0.688. Although this hazard ratio was not statistically significant, the TTR curve difference between Groups A and B was more pronounced than between Groups A and C throughout the course of the 3-year study. Table 3 also shows that Group B demonstrated substantial, although not statistically significant, rates of TTR improvement compared with those of Group A at weeks 48 (35.1%) and 156 (21.8%).

DFS: As shown in Figure 2B, the DFS trends for Group B were more distinct compared with those of Groups A and C throughout the 3-year study. Although the DFS probabilities at the end of the 3-year study of both Groups A (38.5%) and B (49.4%) were lower than the respective probabilities at week 48 (54.1% and 68.4%), the magnitude of the rate of DFS improvement observed

Table 5 Summary of time-to-recurrence, disease-free survival probability, and overall survival results from the follow-up study *n* (%)

Probability	Phase Ⅱ study Week-48		3-yr study Week-156	
	Group A untreated	Group B 160 mg/d	Group A untreated	Group B 160 mg/d
TTR probability[1]	45.9%	29.8%	61.5%	48.1%
Difference		-16.1%		-13.4%
95%CI		-33.6-1.5		-31.5-4.7
Rate of improvement[2]		35.1%		21.8%
P value[3]		0.086		0.187
DFS probability[4]	54.1%	68.4%	38.5%	49.4%
Difference		14.3%		10.8%
95%CI		-3.4-32.0		-7.3-29.0
Rate of improvement		26.4%		28.1%
P value		0.129		0.257
OS probability	90.9%	88.6%	81.0%	82.8%
Difference		-2.3%		1.7%
95%CI		-13.4-8.8		-12.4-15.9
Rate of improvement		-2.5%		2.2%
P value		0.760		1.000

[1,4]Values from Figure 2 respectively; [2]Percent change in endpoint probability of treated group from untreated control; [3]Fisher's exact test. Although not statistically significant, time-to-recurrence (TTR) and disease-free survival (DFS) probabilities and rates of improvements in the 160 mg/d group indicate substantial clinical advantages. Similarities in the two rates of DFS improvement indicate that the clinical benefits of 36 wk of treatment with 160 mg/d PI-88 persisted for up to 3 years. Despite the clinical survival benefits indicated by DFS, overall survival (OS) benefits were inconclusive.

Table 6 Subgroup analyses comparing disease-free survival probabilities of the 160 mg/d group to their respective controls in the phase Ⅱ and follow-up studies

Subgroup analyses	Phase Ⅱ study Week 48		3-yr study Week 156	
	Group A untreated	Group B 160 mg/d	Group A untreated	Group B 160 mg/d
Study cohort				
DFS probability	54.1%	68.4%	38.5%	49.4%
Difference		14.3%		10.8%
95%CI		-3.4-32.0		-7.3-29.0
Rate of improvement[1]		26.4%		28.1%
P value[2]		0.129		0.257
Intermediate-risk group (multiple or single tumor ⩾ 2 cm)				
DFS probability	45.1%	63.8%	33.0%	48.4%
Difference		18.7%		15.4%
95%CI		-0.8-38.2		-3.9-34.7
Rate of improvement		41.5%		46.6%
P value		0.104		0.150
High-risk group (multiple or single tumor ⩾ 2 cm and positive HBV/HCV infection)[3]				
DFS probability	41.4%	64.9%	29.6%	46.4%
Difference		23.5%		16.8%
95%CI		2.0-45.0		-4.4-38.1
Rate of improvement		56.8%		56.8%
P value		0.045		0.163

[1,2] Table 3 footnote 2 and 3; [3] Values from Figure 3. The clinical benefits of 160 mg/d PI-88 were more pronounced in intermediate and high-risk groups. Statistically significant survival benefits were observed in the high-risk group at the end of the phase Ⅱ study. TTR: Time-to-recurrence; DFS: Disease-free survival; OS: Overall survival; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

at week 48 (26.4%) was maintained up to the end of the follow-up study (28.1%).

OS: As shown in Table 5, the OS probabilities of Groups A and B were comparable at the end of the phase Ⅱ and follow-up studies. Although the OS of Group A was higher than that of Group B at week 48, this trend was reversed at week 156.

Subgroup analyses

Table 6 illustrates the effects of PI-88 on patients with tumors and host factors that influence recurrence. Two trends were observed: First, a correlation between DFS improvement and the number of risk factors promoting recurrence was determined. Numerically, this was demonstrated by the increase in DFS improvement in the untreated subgroups from 26.4% to 56.8% at week 48 and from 28.1% to 56.8% at week 156. Similarly, the most significant DFS improvement rate was observed in the high-risk subgroup. Second, the phase Ⅱ cohort exhibited a slightly higher rate of DFS improvement compared with that of the respective 3-year cohorts. Overall, only

the high-risk cohort achieved a statistically significant DFS improvement (56.8%) at the end of the phase Ⅱ study ($P = 0.045$).

The results of the Child-Pugh class A and B subgroup analysis were inconclusive because neither a clinical nor statistical difference was demonstrated (data not shown).

DISCUSSION

The results of this follow-up study corroborated the findings of the phase Ⅱ study: PI-88 administered at 160 mg/d is well-tolerated and may be effective in reducing HCC recurrence. Furthermore, previously observed clinical benefits during the initial 36 wk of active treatment persisted for up to 3 years with minimal AEs. Because numerous premature withdrawals caused by treatment-related toxicities occurred, PI-88 administered at 250 mg/d (Group C) did not confer significant clinical benefits. The following discussion focuses on the 160 mg/d dosage.

Overall, these results suggest that PI-88 administered at 160 mg/d may confer clinical benefits on HCC patients whose tumors have been surgically removed with curative intent. The 36-wk 160 mg/d PI-88 treatment delayed median TTR to over 3 years, and decreased the 3-year TTR probability by 21.8%. In addition to delaying the onset and decreasing the frequency of recurrence, PI-88 treatment also conferred survival advantages; the DFS rate of the 160 mg/d cohort, 49.4%, was the highest rate observed among the cohorts and demonstrated a 28.1% improvement compared with that of the control group. Similar DFS improvements observed at the end of the phase Ⅱ and follow-up studies (26.4% and 28.1%) suggest that the survival benefits of short term PI-88 treatment were maintained for 2 additional years without implementing additional treatment. The OS results, as expected in a small population study for adjuvant cancer therapy, were inconclusive. Although the clinical benefits did not reach statistical significance, active treatment was limited to only 36 wk in the original phase Ⅱ study, and the prolonged use of 160 mg/d PI-88 may result in more pronounced benefits; further trials are necessary to verify this.

Patient stratification observed using subgroup analyses further supported PI-88 efficacy. Because the presence of multiple tumors or a single tumor ⩾ 2 cm was correlated with high degrees of intrahepatic tumor spread and vascular invasion[5], patients with multiple tumors or a single tumor ⩾ 2 cm, together with chronic HBV and HCV infection, are more susceptible to recurrence after surgery. The decreasing DFS probabilities in the untreated cohort exemplify this trend (Table 4). Moreover, the constant DFS probabilities and increasing DFS improvements in the treated intermediate-risk and high-risk groups reinforce the efficacy of PI-88 in reducing recurrence, and also suggest that its effects may be more pronounced in patients with factors that promote HCC recurrence.

The high-risk subgroup exhibited a DFS improvement of 56.8% at the end of the phase Ⅱ study ($P = 0.045$), and this result was statistically significant. Although this rate of DFS improvement was maintained up to the end of this follow-up study, the magnitude of this improvement was not statistically significant ($P = 0.163$). This suggests that the change in DFS improvement is not a reflection of PI-88 treatment efficacy; instead, it is possibly a reflection of the decrease in sample size, which may have prevented clinically significant benefits from reaching statistical significance. Thus, a larger patient population is required in the phase Ⅲ study to verify the statistical significance of the clinical efficacy of PI-88.

As per expert suggestion[8,15], the TTR and OS endpoints were also assessed in this follow-up study. The TTR endpoint effectively revealed the efficacy of PI-88 in delaying recurrence. Although certain regulatory agencies prefer using OS as a measure of efficacy, properly powering OS as an early-stage cancer adjuvant therapy may be difficult for multiple reasons. One reason is that other therapeutic modalities, such as alternative medicine, may confound the efficacy and survival advantages of the treatment under study. Another reason is that, because resection is potentially curative, the extended observation time may have allowed deaths unrelated to the treatment or HCC recurrence to confound the study data. Finally, compliance to PI-88 and access to other therapy or medicine for the treatment of recurrent HCC may also confound the OS. In this observation study, treatment strategy for the recurrent tumor was determined individually by the investigator. Selection bias may significantly influence the overall outcomes of HCC recurrence, which however could not be controlled. Thus, the inconsistent OS probabilities observed in this study may be attributed to these reasons or to the limited sample size.

To investigate the implications of patient heterogeneity, as suggested by Forner and Roayaie[15], subgroup analysis excluding the 4 (2 patients each from Groups A and B) borderline Child-Pugh B patients was performed. Unfortunately, because of the small sample size, the results were inconclusive (Figure 3).

The results of this study were consistent with the known mechanisms of PI-88. As a heparanase inhibitor, PI-88 (1) antagonizes interactions between angiogenic GFs and their receptors; (2) inhibits the release of HS-bound angiogenic and fibroblastic GFs; and (3) inhibits the heparanase degradation of ECM[9]. Collectively, these mechanisms produce the antiangiogenic and antimetastatic effects of PI-88, enabling it to simultaneously reduce intrahepatic metastatic and *de novo* HCC recurrences. However, because the causes of *de novo* recurrences are multifactorial, administering combinational therapy using agents with various mechanisms, such as antiviral or other molecular target agents, may confer even greater clinical benefits[16-18].

In addition to limitations in sample size, additional factors may have precluded the attainment of more statistically significant outcomes in this study. Because active

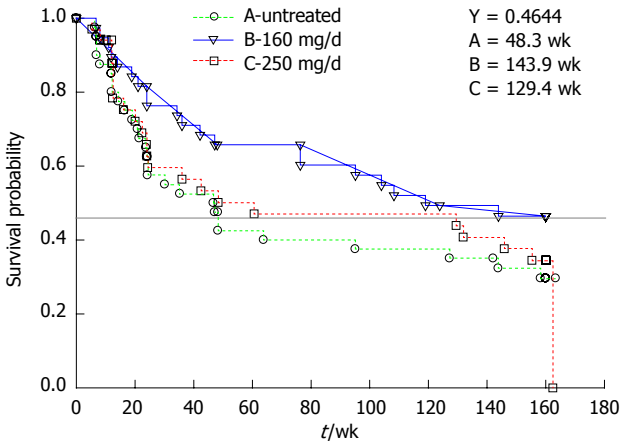

Figure 3 Subgroup analysis of 3-year disease-free survival probability for high-risk subgroups in Groups A, B and C.

cancer treatments are typically administered until either progression or recurrence occurs, prolonged active PI-88 treatment may have conferred even more favorable survival benefits than those observed. Moreover, because resection is potentially curative, extrapolating the length of time required to achieve statistically significant outcomes is difficult, especially because resection and death rates are declining because of technological advances[19,20].

Overall, the findings of this follow-up study are consistent with the findings of the original study, and provide insights that can be used to aid the design of a more conclusive phase Ⅲ trial for the approval of adjuvant PI-88. High tumor recurrence rates and the lack of a standard of care following resection have created a dire need for adjuvant HCC agents; based on the results of the current study, PI-88 is a promising candidate for fulfilling that need.

ACKNOWLEDGMENTS

We extend our thanks to the researchers who participated in this PI-88 HCC phase Ⅱ study: Lin YJ, Shan YS, and Chou TC of National Cheng Kung University Hospital, Tainan, Taiwan; and Tsai CC and Wang BW of Kaohsiung Veterans General Hospital, Kaohsiung, Taiwan. We are indebted to James Wang and Angela Tseng for their statistical assistance. We would also like to thank our study coordinators Chen CY, Lee R, and Lai R for their contributions to this follow-up trial.

COMMENTS

Background

Hepatocellular carcinoma (HCC) recurrence is common after curative resection. There remains a dire clinical need for an adjuvant therapy to reduce the risk of postresection HCC recurrence. A phase Ⅱ clinical trial demonstrated that heparanase inhibitor PI-88 at 160 mg/d is safe and promising in reducing HCC recurrence for up to one year following curative resection.

Research frontiers

Considering the ability of PI-88 to perform these dual functions (antiangiogenesis and antimetastasis), PI-88 can potentially suppress HCC recurrences. This observation study aims to investigate the longterm benefit of PI-88 as an adjuvant therapy for HCC recurrence.

Innovations and breakthroughs

This observational follow-up study extended the follow-up period to 3 years. PI-88 at 160 mg/d delayed the onset and frequency of HCC recurrence, and provided a clinically significant survival advantage for up to 3 years after treatment. Subgroup analyses revealed that administering PI-88 at 160 mg/d conferred the most significant survival advantage for patients at high risk of recurrence.

Applications

The findings of this follow-up study provide insights that can be used to aid the design of a more conclusive phase Ⅲ trial for the approval of adjuvant PI-88.

Terminology

As a heparanase inhibitor, PI-88 (1) antagonizes interactions between angiogenic growth factors and their receptors, (2) inhibits the release of heparin sulfate-bound angiogenic and fibroblastic growth factors, and (3) inhibits the heparanase degradation of extracellular matrix. Collectively, these mechanisms produce the antiangiogenic and antimetastatic effects of PI-88, enabling it to simultaneously reduce intrahepatic metastatic and *de novo* HCC recurrences.

Peer review

The authors present an important follow-up study of outcomes following PI-88 treatment as adjuvant therapy for hepatocellular carcinoma. This study was well done and well-presented.

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P- Reviewer: Lin J, Sangro B, Yanev SG **S- Editor**: Gou SX
L- Editor: A **E- Editor**: Zhang DN

World Journal of Gastroenterology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i32.11394

World J Gastroenterol 2014 August 28; 20(32): 11394-11399
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

OBSERVATIONAL STUDY

Utility of the Asia-Pacific colorectal screening scoring system and the presence of metabolic syndrome components in screening for sporadic colorectal cancer

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Supported by Capital Health Research and Development of Special, No. 2011-4022-06
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Received: January 22, 2014 Revised: April 4, 2014
Accepted: April 30, 2014
Published online: August 28, 2014

Abstract

AIM: To determine the utility of the Asia-Pacific colorectal screening (APCS) scoring system and metabolic syndrome components in individual screening for sporadic colorectal cancer.

METHODS: The subjects were patients admitted to the Peking University People's Hospital for colonoscopy between October 2012 and July 2013. Clinical information, including patient willingness to undergo colonoscopy, medical history, endoscopic findings, histology, and other information, was collected, and the patients were grouped according to APCS scores and the presence of metabolic syndrome components. Colorectal tumor detection rates were compared between the groups.

RESULTS: A total of 219 patients were included in the study, 108 were male and 111 were female, resulting in a male-to-female ratio of 1:1.03. The average age of the patients was 56.8 ± 13.7 years. According to APCS scores, 88 (40.2%) patients were included in the average-risk (AR) group, 113 (51.6%) patients were included in the moderate-risk (MR) group, and 18 (8.2%) patients were included in the high-risk (HR) group. Colorectal tumors were detected in 69 (31.5%) subjects, and the detection rates in the AR, MR, and HR groups were 15.9%, 36.3%, and 77.8%, respectively. The difference in the detection rates between the three groups was statistically significant ($P < 0.01$). The combined detection rate of colorectal tumors in the APCS MR and HR groups was 42.0%. However, patients in the MR and HR groups who presented with metabolic syndrome components, in particular obesity, exhibited a significantly higher colorectal tumor detection rate (59.5%) than did those without these components (19.2%, $P < 0.01$) and those who underwent colonoscopy because of doctor's recommendation (36.5%, $P < 0.01$).

CONCLUSION: The APCS scoring system can be used in individual screening for sporadic colorectal cancer. The combined use of APCS scores and the metabolic syndrome components, in particular obesity, will significantly improve the efficacy of individual colorectal cancer screening.

Key words: Asia-Pacific colorectal screening scoring system; Metabolic syndrome; Obesity; Colorectal cancer; Individual screening

Core tip: This study assessed the utility of the Asia-Pacific colorectal screening (APCS) scoring system and the presence of metabolic syndrome components in outpatient screening for colorectal cancer (CRC) by stratifying individuals according to these parameters. The APCS scoring system can be used in individual screening for sporadic CRC. The combined use of APCS scores and the metabolic syndrome components, in particular obesity, will significantly improve the efficacy of colorectal cancer screening.

Wang JY, Li ZT, Zhu YM, Wang WC, Ma Y, Liu YL. Utility of the Asia-Pacific colorectal screening scoring system and the presence of metabolic syndrome components in screening for sporadic colorectal cancer. *World J Gastroenterol* 2014; 20(32): 11394-11399 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11394.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11394

INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignant tumors worldwide[1-3]. Both the incidence and associated mortality rates of CRC in China have increased because of improved standards of living and changes in eating habits[4]. In most cases, CRC is sporadic and develops from adenomas. As effective CRC prevention methods are lacking, the best method to prevent the morbidity associated with CRC is to improve the detection rate of early-stage disease. A growing body of evidence has shown that detection and elimination of lesions at the precursor or early stage can reduce both the incidence of and mortality from CRC[5]. The decreased incidence of CRC in the United States over the past 2 decades has been attributed by some efficient CRC screening[6].

Many studies have recommended screening programs for CRC[7-9]. Among them, the Asia-Pacific colorectal screening[2] (APCS) system, reported in 2011, uses age, gender, a family history of colorectal tumors, and smoking history as the factors to calculate scores. Individuals are categorized into 3 groups according to these scores: the average-risk (AR), moderate-risk (MR), and high-risk (HR) groups. Colonoscopy is recommended for individuals in the HR group. The APCS scoring system is simple and convenient for outpatient screening; however, it was proposed only recently, and therefore, its effectiveness requires further evaluation. Because the APCS system was established using asymptomatic individuals, whether it is useful for individual screening in symptomatic outpatients was discussed in this study.

Metabolic syndrome (MS) comprises the metabolic risk factors of cardiovascular disease and type 2 diabetes, which mainly include obesity, dyslipidemia, hypertension, hyperglycemia, and insulin resistance[10]. Because of the adoption of Western dietary habits and life-style, many Asian countries have witnessed a substantial increase in the prevalence of obesity and MS over the past few decades[11,12].

A large number of epidemiological studies have shown that MS components are closely related to the occurrence of colorectal tumors[13-17]. However, the APCS scoring system does not incorporate MS components. Therefore, in this study, we assessed the utility of the APCS system and the presence of MS components in outpatient screening for CRC by stratifying individuals according to these parameters.

MATERIALS AND METHODS

Subjects

We selected patients who were admitted to the Peking University People's Hospital for colonoscopy between October 2012 and July 2013. The exclusion criteria were as follows: age less than 20 years or more than 90 years; familial adenomatous polyposis or hereditary non-polyposis CRC; and inflammatory bowel disease.

The study was approved by the Ethical Committee of Peking University People's Hospital. All the patients enrolled in the study were well informed and signed the informed consent.

Methods

We collected the clinical information of patients, including gender; age; body mass index (BMI); history of hypertension, high blood glucose levels, dyslipidemia, alcohol consumption, smoking, and cancer; and the history of CRC in immediate family members. Additionally, we determined the reasons for which patients underwent colonoscopy: whether the patient volunteered to undergo these examinations or whether these examinations were recommended by their doctors.

The colonoscopy findings and tumor pathology results were reviewed. Colorectal tumors were classified as non-advanced tumors (tubular adenoma with a maximum diameter < 1 cm, without severe dysplasia) or advanced tumors (tubular adenoma with a maximum diameter ≥ 1 cm, villous or tubulovillous adenoma, adenoma with severe dysplasia, or cancer).

The subjects were divided into the AR, MR, and HR groups according to APCS scores, which were assigned as follows: age: < 50 years = 0 points, 50-69 years = 1 point, ≥ 70 years = 2 points; gender: female = 0 points, male = 1 point; immediate family member with CRC: no = 0 points, yes = 1 point; and smoking status: no smoking history = 0 points, current or former smoker = 1 point. On the basis of the cumulative scores, patients were classified into the following groups: AR = 0-1, MR = 2-3, and HR = 4-7.

In this study, the MS components and related diseases that were evaluated were obesity (BMI ≥ 25 kg/m^2), hyperlipidemia, hypertension, and diabetes. According to

Table 1 Comparison of the colorectal tumor detection rates in patients in the different Asia-Pacific colorectal screening risk groups *n* (%)

Groups	AR (*n* = 88)	MR (*n* = 113)	HR (*n* = 18)
Colorectal tumor	14 (15.9)	41 (36.3)[b]	14 (77.8)[bd]
Non-advanced tumor	12 (13.6)	30 (26.5)[a]	9 (50)[bc]
Advanced tumor	2 (2.3)	11 (9.7)[a]	5 (27.8)[bc]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* AR group; [c]$P < 0.05$, [d]$P < 0.01$ *vs* MR group. AR: Average-risk; MR: Moderate-risk; HR: High-risk.

the presence of MS components and related disorders, individuals in the APCS MR and HR groups were further stratified and colorectal tumor detection rates were compared between individuals who did and those who did not have these MS components and related diseases to determine their utility in CRC screening.

Statistical analysis

All data were analyzed using IBM SPSS Statistics 20 software (SSPS, Inc., Chicago, IL, United States). Continuous variables with a normal distribution were presented as means ± SD. Categorical data were presented as percentages (%) and were analyzed using the χ^2 test or Fisher's exact test, with a significance level of $\alpha = 0.05$. A P value of < 0.05 was considered statistically significant.

RESULTS

General characteristics

Among the 219 enrolled patients, 108 were male and 111 were female, resulting in a male-to-female ratio of 1:1.03. The average age of the patients was 56.8 ± 13.7 years. Among the 219 patients, 52 (23.8%) had a history of colorectal polyps and 4 had a history of CRC. Colorectal tumors were detected in 69 patients (31.5%); of these, 14 (15.9%) were in the AR group, 41 (36.3%) were in the MR group, and 14 (77.8%) were in the HR group. Further, 51 patients (23.3%) had non-advanced tumors and 18 (8.2%) had advanced tumors, including 9 cases of invasive carcinoma.

Effect of stratification according to APCS scores on the colorectal tumor detection rate

According to the APCS criteria, of the 219 patients, 88 (40.2%) were in the AR group, 113 (51.6%) were in the MR group, and 18 (8.2%) were in the HR group. The colorectal tumor detection rates for the AR, MR, and HR groups were 15.9%, 36.3%, and 77.8%, respectively, and the differences between the groups were statistically significant (Table 1). The combined detection rate of the MR and HR groups was 42.0%.

Effect of the presence of MS components on the colorectal tumor detection rate

A sub-group analysis was performed on the 131 patients in the APCS MR and HR groups. Based on the presence of MS components and related diseases, these patients were divided into 3 groups: group A (control group), BMI < 25 kg/m^2 and no MS components; group B, BMI $\geqslant 25$ kg/m^2 with 1 or no MS components; and group C, BMI $\geqslant 25$ kg/m^2 with 2-3 MS components. The colorectal tumor detection rates were as follows: group A, 19.2% (10/52); group B, 56.4% (22/39); group C, 62.5% (25/40); and group B + C, 59.5% (47/79). Statistical analysis revealed that the values of groups B and C were significantly different from that of group A; however, the difference between groups B and C was not statistically significant (Table 2).

Table 2 Effect of the presence of metabolic syndrome components on the colorectal tumor detection rate *n* (%)

Groups	A (*n* = 52)	B (*n* = 39)	C (*n* = 40)	B + C (*n* = 79)
Colorectal tumor	10 (19.2)	22 (56.4)[b]	25 (62.5)[b]	47 (59.5)[b]
Non-advanced tumor	9 (17.3)	14 (35.9)[a]	16 (40)[a]	30 (38.0)[a]
Advanced tumor	1 (1.9)	8 (20.5)[b]	9 (22.5)[b]	17 (21.5)[b]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* group A.

Table 3 Comparison of the colorectal tumor detection rates for different screening programs *n* (%)

Groups	Patient volunteered (D) (*n* = 41)	Doctor recommended (E) (*n* = 126)	APCS MR/HR (F) (*n* = 131)	APCS MR/HR with obesity (G) (*n* = 79)
Colorectal tumor	6 (14.6)	46 (36.5)[b]	55 (42.0)[b]	47 (59.5)[bde]
Non-advanced tumor	5 (12.2)	33 (26.2)	39 (29.8)[a]	30 (38.0)[b]
Advanced tumor	1 (2.4)	13 (10.3)	16 (12.2)	17 (21.5)[bc]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* group D; [c]$P < 0.05$, [d]$P < 0.01$ *vs* group E; [e]$P < 0.05$ *vs* group F. APCS: Asia-Pacific colorectal screening; MR: Moderate-risk; HR: High-risk.

Comparison of the colorectal tumor detection rates for different screening programs

On the basis of their reasons for undergoing colonoscopy, patients were divided into groups D (those who volunteered to undergo colonoscopy) and E (those who underwent colonoscopy based on their doctor's recommendation), and the colorectal tumor detection rates were compared between the two groups. These data were also compared to those of the APCS MR/HR group (group F) and the APCS MR/HR group with obesity (BMI $\geqslant 25$ kg/m^2 with or without other MS components; group G). The colorectal tumor detection rate was significantly higher in group E than in group D ($P < 0.01$), whereas this rate was significantly higher in group G than in group E ($P < 0.01$) and in group F ($P < 0.05$; Table 3).

DISCUSSION

Researchers at the National University of Singapore

conducted a study on tertiary hospitals in 11 Asian cities, including 2752 asymptomatic individuals who underwent screening colonoscopy. The researchers developed the APCS scoring system based on the results of multivariate logistic regression analysis of the risk factors for colorectal tumors[9]. In the validation group, the rates of advanced neoplasia in the AR, MR, and HR groups were 1.3%, 3.2%, and 5.2%, respectively. Patients in the MR and HR groups had a 2.6-fold (95%CI: 1.1-6.0) and 4.3-fold (95%CI: 1.8-10.3) higher rate of advanced neoplasia, respectively, than patients in the AR group. In our study, we observed colorectal tumor detection rates of 15.9%, 36.3%, and 77.8%, respectively, for the AR, MR, and HR groups, and the detection rates for advanced colorectal tumor were 2.3%, 9.7%, and 27.8% in these groups, respectively. The differences in the detection rates between the 3 groups were statistically significant, suggesting that APCS scoring system was useful in colorectal tumor screening for symptomatic outpatients, although the APCS scoring system was established using asymptomatic individuals. In addition, the APCS scoring system is simple and convenient for clinical use. In this study, the colorectal tumor detection rate was significantly higher than that in the APCS study; a possible reason for this difference is that many patients who visited the clinic had related symptoms and risk factors such as a history of colorectal polyps or CRC. Patients with a history of colorectal polyps might have recurrence rates exceeding 55.7%[15], and therefore, the colorectal tumor detection rate is higher in those patients than in the general population.

Numerous studies have indicated that MS components and related diseases are significantly associated with the development of colorectal tumors, and MS is a significantly independent element that influences the survival of the CRC[14-18]. Obesity is a key component of MS[19], and systematic reviews[20,21] revealed that obesity is a statistically significant risk factor for CRC. Therefore, we further stratified patients in the MR and HR groups according to these risk factors. When patients in the MR/HR group were stratified according to the presence of obesity, the colorectal tumor detection rate significantly increased (59.5% *vs* 19.2% for the MR/HR group without obesity, $P < 0.01$). Thus, MS components, especially obesity, are closely related to the onset of colorectal tumors.

Our previous studies[15,16] demonstrated that an increase in the number of MS components is related to an increased proportion of advanced colorectal tumors among all colorectal tumors. The number of MS components is also related to tumor recurrence. Patients with colorectal tumors that are not associated with any MS components have a recurrence rate of 18.42% within 1-3 years after initial treatment, whereas those with 1, 2, or $\geqslant$ 3 components have recurrence rates of 59.52%, 75%, and 77.78%, respectively. This finding suggests that the number of MS components is positively correlated with the risk of colorectal tumor development. This study revealed that the colorectal tumor detection rate increased as the number of MS components increased; however, this increase did not reach statistical significance, which was probably due to the small sample size.

Individual screening, also known as opportunistic screening, comprises clinical screening and a face-to-face examination. This may be performed when patients request screening from doctors or doctors prescribe screening tests on the basis of the presence of risk factors. In fact, most early CRC diagnoses in China result from individual screening recommended by doctors in hospital[22]; however, neither doctors nor patients have clear guidelines to follow. In China, the CRC screening program involves screening methods such as the sequential fecal occult blood initial screening method[23]. Even with immunoassays of fecal occult blood, the detection rate of early-stage CRC is relatively low[23,24]. Thus, in CRC screening programs, a survey of high-risk factors for CRC should be emphasized, so that patients in the high-risk group can undergo screening colonoscopy without delay.

According to former studies, the extent of knowledge regarding CRC that primary care doctors and the general population have greatly influences compliance with CRC screening[25,26]. Given the limited medical resources, we should identify high-risk patients for prior colonoscopy screening. In the clinic, doctors' decisions are greatly influenced by personal experiences. Most doctors are not familiar with specific CRC screening principles, and many patients fear colonoscopy. This study illustrated that the combined use of the APCS score and the presence of MS components such as obesity had a significantly better effect on CRC screening than did physician advice or the patient's willingness to undergo colonoscopy. We suggest that the effects of the APCS scores as well as those of MS components on CRC onset be advocated in doctors and among the general population. Increased compliance with CRC screening should be ensured among patients in the APCS MR/HR group, especially among those with MS components, particularly obesity, to increase the efficiency of individual CRC screening programs and promote the accumulation of experiences and data that will provide evidence for improving CRC screening in China.

COMMENTS

Background

Colorectal cancer (CRC) is one of the most common malignant tumors worldwide. The best method to prevent the morbidity associated with CRC is to improve the detection rate of early-stage disease. The Asia-Pacific colorectal screening (APCS) system uses age, gender, a family history of colorectal tumors, and smoking history as the factors to calculate the risk of colorectal tumors. However, the APCS scoring system does not incorporate metabolic syndrome, which has been proved closely related to the occurrence and development of colorectal tumors.

Research frontiers

There are a large number of studies aiming to reveal the risk factors of CRC, including but not limited to age, gender, smoking, a family history of colorectal tumors and metabolic syndrome. Accordingly, many screening models for CRC

have been recommended.

Innovations and breakthroughs

This study assessed the efficacy of APCS scoring system and the combined use of metabolic syndrome in individual screening for sporadic CRC.

Applications

The combined use of APCS scores and the metabolic syndrome components, in particular obesity, may significantly improve the efficacy of individual CRC screening, and has clinical significance.

Terminology

Individual screening, also known as opportunistic screening, is a new model based on clinical practice. It may be performed when patients request screening from doctors or doctors prescribe screening tests on the basis of the presence of risk factors.

Peer review

The APCS scoring system can be used in individual screening for sporadic colorectal cancer. The combined use of APCS scores and the metabolic syndrome components, in particular obesity, will significantly improve the efficacy of colorectal cancer screening. This is a potentially important study, especially in the Asian population, and has clinical significance. The manuscript is well written and clear.

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P- Reviewer: Kim JS, Salkic NN, Xie K **S- Editor**: Qi Y
L- Editor: O'Neill M **E- Editor**: Wang CH

World Journal of Gastroenterology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i32.11400

World J Gastroenterol 2014 August 28; 20(32): 11400-11405
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

OBSERVATIONAL STUDY

Clinical efficacy of tolvaptan for treatment of refractory ascites in liver cirrhosis patients

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Author contributions: Zhang X and Wang SZ contributed equally to the manuscript; Ding HG, Zhang X, Wang SZ and Zheng JF analyzed the data; Zhang X, Wang SZ and Ding HG wrote the manuscript; Ding HG was also responsible for this project and the final revised manuscript; The other authors contributed to data acquisition, patient care and follow up.

Supported by The Program of Beijing Science and Technology Commission, No. D131100005313004; and the Beijing High-Level Talent Academic Leader/Personnel Training Programs awarded to Ding HG, 2011-2-19; Li B, 2013-3-072; and Li L, 2013-3-073

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Received: February 8, 2014 Revised: April 11, 2014
Accepted: May 12, 2014
Published online: August 28, 2014

Abstract

AIM: To evaluate the efficacy and safety of tolvaptan to treat refractory ascites in decompensated liver cirrhosis patients with or without further complications, such as hepatorenal syndrome and/or hepatocellular carcinoma.

METHODS: Thirty-nine patients (mean age 55 years, males: 32) with decompensated liver cirrhosis and refractory ascites were enrolled. All patients received a combination of tolvaptan (15 mg/d for 5-14 d) and diuretics (40-80 mg/d of furosemide and 80-160 mg/d of spironolactone). The etiology of cirrhosis included hepatitis B (69.2%), hepatitis C (7.7%) and alcohol-induced (23.1%). Changes in the urine excretion volume, abdominal circumference and edema were assessed. The serum sodium levels were also measured, and adverse events were recorded. A follow-up assessment was conducted 1 mo after treatment with tolvaptan.

RESULTS: Tolvaptan increased the mean urine excretion volume (1969.2 ± 355.55 mL *vs* 3410.3 ± 974.1 mL, $P < 0.001$), and 89.7% of patients showed improvements in their ascites, 46.2% of whom showed significant improvements. The overall efficacy of tolvaptan in all patients was 89.7%; the efficacies in patients with hepatocellular carcinoma and hepatorenal syndrome were 84.2% and 77.8%, respectively. The incidence of hyponatremia was 53.8%. In patients with hyponatremia, the serum sodium levels increased after tolvaptan treatment (from 128.1 ± 4.22 mEq/L *vs* 133.1 ± 3.8 mEq/L, $P < 0.001$). Only mild drug-related adverse events, including thirst and dry mouth, were observed.

CONCLUSION: Tolvaptan is a promising aquaretic for the treatment of refractory ascites in patients with decompensated liver cirrhosis.

Key words: Tolvaptan; Refractory ascites; Hyponatremia; Decompensation; Liver cirrhosis

Core tip: This study showed that tolvaptan, a new aquaretic drug, is an effective and safe potential treatment for refractory ascites in patients with decompensated liver cirrhosis, especially in patients with further complications, such as hepatorenal syndrome and/or hepatocellular carcinoma. Tolvaptan significantly increased the serum sodium levels in patients with hyponatremia.

Zhang X, Wang SZ, Zheng JF, Zhao WM, Li P, Fan CL, Li B,

Dong PL, Li L, Ding HG. Clinical efficacy of tolvaptan for treatment of refractory ascites in liver cirrhosis patients. *World J Gastroenterol* 2014; 20(32): 11400-11405 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11400.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11400

INTRODUCTION

Ascites is one of the most common complications of liver cirrhosis[1]. Refractory ascites occur in 15%-20% of all ascites patients and is defined either as an unresponsiveness to restrictions on salt intake and high-dose diuretics, or as a recurrence occurring rapidly within 4 wk post-therapy, according to the American Association for the Study of Liver Diseases (AASLD) guidelines[2]. Refractory ascites are associated with a poor prognosis, and are difficult to treat because of limited treatment options[3-6].

Tolvaptan is a new, oral, selective vasopressin V_2-receptor antagonist approved for treating hypervolemic and euvolemic hyponatremia[7,8]. Blockage of V_2-receptors by tolvaptan prevents the insertion of aquaporin-2 water channels into the apical cell membrane of the collecting duct, increasing free water excretion without significantly affecting urinary sodium and potassium excretion. As a result, there is reduced water retention with elevated serum sodium levels. The mechanism of action of tolvaptan indicates that it is effective for treating hyponatremia and has a significant role in promoting aquaresis.

Numerous studies have reported the efficacy and safety of tolvaptan for treating ascites and edema in patients with decompensated cirrhosis[8-11]. However, the efficacy and safety of this drug for treating refractory ascites in cirrhotic patients remains unknown. Furthermore, the use of tolvaptan in the subset of patients with complications, such as hepatorenal syndrome and/or hepatocellular carcinoma, has not been explored previously.

Our principal objective was to conduct an observational study to examine the efficacy and safety of tolvaptan to treat refractory ascites and edema in decompensated cirrhotic patients with or without additional complications.

MATERIALS AND METHODS

Study design

A single center, open-label, observational study was conducted in China between May 2012 and July 2013. Patients were recruited between May 2012 and March 2013.

Inclusion criteria

Candidates were selected for study inclusion if they met the criteria for cirrhosis and refractory ascites. A diagnostic work-up for decompensated liver cirrhosis was performed, including assessment of the clinical manifestations, physical examination and laboratory tests. The inclusion criteria were as follows: (1) history of chronic hepatitis and/or signs with various causes; (2) abnormal liver function accompanied by portal hypertension, such as ascites, encephalopathy or esophageal or gastric variceal bleeding; and (3) B-ultrasound scanning (LOGIQ9; GE Company, Fairfield, United States) and four-phase multidetector computed tomography (CT) scan (GE HISPEED DXI; GE Company) results consistent with the signs of liver cirrhosis. Patients with hepatocellular carcinoma were diagnosed using CT or dynamic contrast-enhanced magnetic resonance imaging, in accordance with the diagnostic criteria recommended in the 2010 AASLD guidelines[12]. The definition of refractory ascites underwent a minor revision according to the 2012 AASLD guidelines[2]. Namely, refractory ascites were defined if it was not satisfactorily controlled after a patient had either (1) 1 wk of sodium intake restrictions (< 6 g/d), intermittent albumin infusion (10-20 g per treatment) and high doses of diuretics (more than 160 mg/d of furosemide and 200 mg/d of spironolactone); or (2) 2 wk of therapeutic paracentesis (3000-5000 mL per treatment). Hepatic encephalopathy was assessed in accordance with the guidelines developed by the American Gastroenterological Association[13]. Patients were diagnosed with hepatorenal syndrome in accordance with the International Ascites Club guidelines[14]. Patients were excluded if they had severe cardiovascular, pulmonary, cerebral or hematological complications or severe mental illness.

Ethics

The ethics committee of Beijing You'an Hospital, Capital Medical University approved the current study (protocol number 2011-015) and it was performed in accordance with the ethical standards set forth in the 1964 Declaration of Helsinki and its later amendments. Informed consent was obtained from patients and their families before study participation.

Therapeutic protocol

All patients received oral tolvaptan (15 mg/d for 5-14 d) in addition to a concurrent treatment regimen of sodium intake restrictions (< 6 g/d), intermittent albumin infusion (10-20 g per treatment) and standard diuretic therapy (40-80 mg/d of furosemide and 80-160 mg/d of spironolactone). Patients with abdominal infections were given antibiotics before tolvaptan treatment, and patients with coexisting hepatic encephalopathy also underwent a routine treatment regimen of lactulose. A follow-up assessment was conducted 1-mo post-tolvaptan treatment for all patients.

An electrochemiluminescence immunoassay was used to detect serum markers for hepatitis B virus and hepatitis C virus, in accordance with the manufacturer's protocol (Roche E170 modular immunoassay analyzer, Roche Diagnostics, Mannheim, Germany). An automatic biochemical analyzer (AU5400, Olympus, Japan) was used to test liver and renal function, including the serum sodium and potassium levels, serum alanine aminotransferase, aspartate aminotransferase, total bilirubin, albumin, creatinine and urea nitrogen.

Table 1 Definitions for grades representing the measures of improvement for the urine excretion volume, ascites and edema

	Measure of improvement		
	Significant improvement	Improvement	No improvement
Grade	A	B	C
Volume of urine excretion	Increased by > 1000 mL/24 h	Increased by 500-1000 mL/24 h	Increased by < 500 mL/24 h
Abdominal circumference	Decreased by > 2 cm	Decreased by 0-2 cm	No change
Edema (lower extremities)	A lack of visible pitting	Presence of visible pitting	Distinctly visible pitting

Table 2 Baseline characteristics of the cirrhotic patients with refractory ascites included in the study *n* (%)

Baseline characteristics		Value
Total number of patients		39
Age (yr)		55.0 ± 12.4
Sex	Male	32 (82.0)
	Female	7 (17.9)
Underlying liver disease	Cirrhosis	20 (51.3)
	Cirrhosis with hepatocellular carcinoma	19 (48.7)
Liver disease etiology	HBV infection	27 (69.2)
	HCV infection	3 (7.69)
	Alcoholic cirrhosis	9 (23.1)
Coexisting hepatorenal syndrome	Type 1	2 (5.13)
	Type 2	7 (17.9)
Coexisting hepatic encephalopathy		13 (33.3)
Child-Pugh score	Class C	39 (11.8 ± 1.5)
MELD score		39 (37.5 ± 5.6)
Coexisting diabetes		9 (23.1)
Hyponatremia	Yes	21 (53.8)
	No	18 (46.2)

Data are expressed as absolute numbers (percentage) or mean ± SD. HBV: Hepatitis B virus; HCV: Hepatitis C virus; MELD: Model for end-stage liver disease.

Efficacy assessment

For ascites, the urine volume was measured over 24 h; we also measured the abdominal circumference and edema of the lower extremities, which were assessed in the morning while the participants had an empty stomach. The overall efficacy of tolvaptan for treating ascites was assessed using a set of three evaluation indicators, which are shown in Table 1.

Survival

The number of patients who survived after 1 mo was recorded to examine the relationship between the short-term correction of hyponatremia and prognostic improvement.

Safety assessment

Patients were monitored throughout the study period, and any occurrences of adverse events and deaths were recorded.

Statistical analysis

Parametric data are expressed as the mean ± standard deviation (mean ± SD) and were assessed using two-tailed *t*-tests. Levene's test for equality of variance was conducted concurrently for all *t*-tests. Categorical data were compared using the Pearson's χ^2 test. The Kaplan-Meier method was used to estimate the 1-mo cumulative patient survival, and between-group differences were tested for significance using the log-rank test. *P*-values < 0.05 were considered statistically significant. All statistical analyses were performed using IBM SPSS Statistics software (IBM, version 12.0).

RESULTS

Patient characteristics

Thirty-nine patients who met the study's eligibility requirements were included. The demographics and other baseline characteristics of these patients are shown in Table 2.

Ascites and edema

The administration of tolvaptan resulted in a significant increase in the mean urine excretion volume, from 1969.2 ± 355.55 mL pre-treatment to 3410.3 ± 974.1 mL post-treatment [$t(48) = -8.679$, $P < 0.001$; $n = 39$]. Levene's test indicated that there were unequal variances in the data sets ($F = 27.115$, $P < 0.001$); as a result, the degrees of freedom were adjusted from 76 to 48. The combination of tolvaptan with diuretics effectively increased the urine output in 89.7% of patients with refractory ascites (Table 3). The abdominal circumference was reduced in 82% of patients, and edema was also improved in 91.7% of patients (Table 3).

The overall efficacy of tolvaptan was 89.7% ($n = 35$) in all patients, and 46.2% ($n = 18$) of these patients had significant improvement (Table 3 and Figure 1). Subgroup analyses indicated that the overall efficacy of tolvaptan in patients with coexisting hepatocellular carcinoma was 84.2% ($n = 19$) and that the efficacy for patients with coexisting hepatorenal syndrome was 77.8% ($n = 9$; Table 3 and Figure 1). Tolvaptan was not effective to treat refractory ascites in patients with coexisting Type 1 hepatorenal syndrome.

Hyponatremia

The incidence of hyponatremia (defined as a serum sodium concentration < 135 mEq/L) was 53.8% (21 of 39 patients) in cirrhotic patients with refractory ascites. Tolvaptan caused a significant increase in the serum sodium concentration in patients with hyponatremia (from 128.1 ± 4.22 to 133.1 ± 3.8 mEq/L; Figure 2). There was

Table 3 Summary of the improvement in the urine excretion, ascites and edema after tolvaptan treatment, according to grading criteria, and in the overall improvement *n* (%)

	n	Significant improvement	Improvement	No improvement
Urine excretion	39	26 (66.7)	9 (23.1)	4 (10.3)
Abdominal circumference	39	19 (48.7)	13 (33.3)	7 (17.9)
Edema (lower extremities)	24	17 (70.8)	5 (20.8)	2 (8.3)
Overall improvement for all patients[1]	39	18 (46.2)	17 (43.6)	4 (10.2)
Overall improvement in patients with coexisting hepatocellular carcinoma[1]	19	9 (47.4)	7 (36.8)	3 (15.8)
Overall improvement in patients with coexisting hepatorenal syndrome (Type 1)[1]	2	0 (0)	0 (0)	2 (100.0)
Overall improvement in patients with coexisting hepatorenal syndrome (Type 2)[1]	7	2 (28.6)	5 (71.4)	0 (0)

[1]Overall improvement definitions: Significant improvement = grade of A for urine excretion, ascites and edema; Improvement = grade of B for urine excretion and a grade of improvement for ascites or edema; No improvement = grade of C for urine excretion.

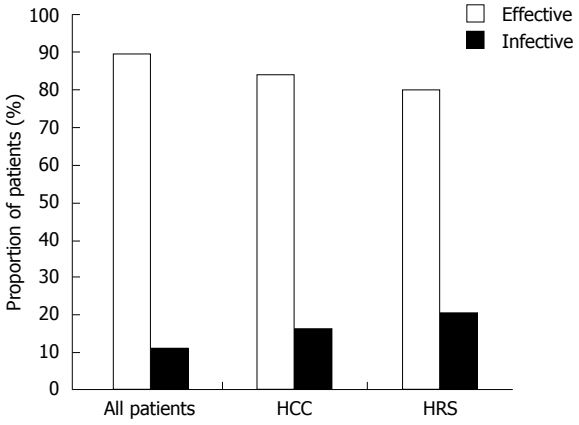

Figure 1 Overall efficacy of 15 mg/d tolvaptan. Overall efficacy of 15 mg/d tolvaptan for 5-14 d in all patients as well as in subgroups of patients with coexisting hepatocellular carcinoma (HCC) and hepatorenal syndrome (HRS) post-tolvaptan treatment.

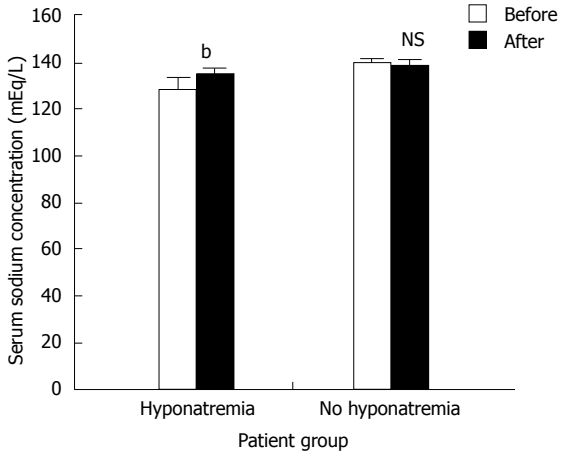

Figure 2 The effects of tolvaptan on serum sodium (mean ± SE) in patients with and without hyponatremia (*n* = 21 and *n* = 18, respectively). Tolvaptan (15 mg/d, 5-14 d) treatment (black column) significantly increased serum sodium concentration [t (40) = -4.029, $^bP < 0.01$ *vs* before treatment group] in patients with hyponatremia, but not in patients without hyponatremia [t (32) = 1.545, $P > 0.05$ (NS)]. NS: Not significant.

no significant change in the serum sodium concentration for patients lacking hyponatremia after tolvaptan treatment (137.8 ± 3.02 mEq/L before treatment and 136.9 ± 3.18 mEq/L after treatment; Figure 2). There was no significant relationship between the short-term correction of hyponatremia and the 1-mo patient survival rate [Figure 3; χ^2 (2, n = 39) = 0.454, $P > 0.05$].

Adverse events

Mild adverse events (thirst and dry mouth) associated with tolvaptan treatment were reported in four and two patients, respectively. No other drug-related adverse events or liver function abnormalities were observed in this study. Improvements were observed in 11 of 13 patients who had coexisting hepatic encephalopathy. In patients with hepatorenal syndrome, five of seven patients with Type 2 syndrome showed improvements during treatment, while two patients with coexisting Type 1 syndrome experienced continuous deterioration of their renal function.

DISCUSSION

The current theory for the development of ascites is complex and has recently evolved to include previously proposed mechanisms[5]. However, to fully understand how tolvaptan affects refractory ascites, the pathogenesis of ascites needs to be briefly explored.

The formation of ascites involves an increase in the hepatic portal vein pressure, which may cause systemic arterial vasodilation, resulting in a decrease in the effective blood volume[15-17]. This decrease in blood volume triggers the activation of the sympathetic nervous system and the renin-angiotensin system; the resultant cascade of events causes the release of arginine vasopressin (AVP, which is alternatively termed antidiuretic hormone). AVP plays a critical role in water reabsorption in the renal collecting ducts[5,8]. However, as mentioned previously, the treatments for refractory ascites are limited. A diet of controlled salt intake, adequate diuretics treatment, large-volume paracentesis, transjugular intrahepatic portosystemic shunting and liver transplantation are the current clinical recommendations[3,18] However, targeting the AVP mechanism presents a potential novel treatment approach for refractory ascites.

Tolvaptan improves ascites and edema in patients with decompensated liver disease, but its efficacy has not

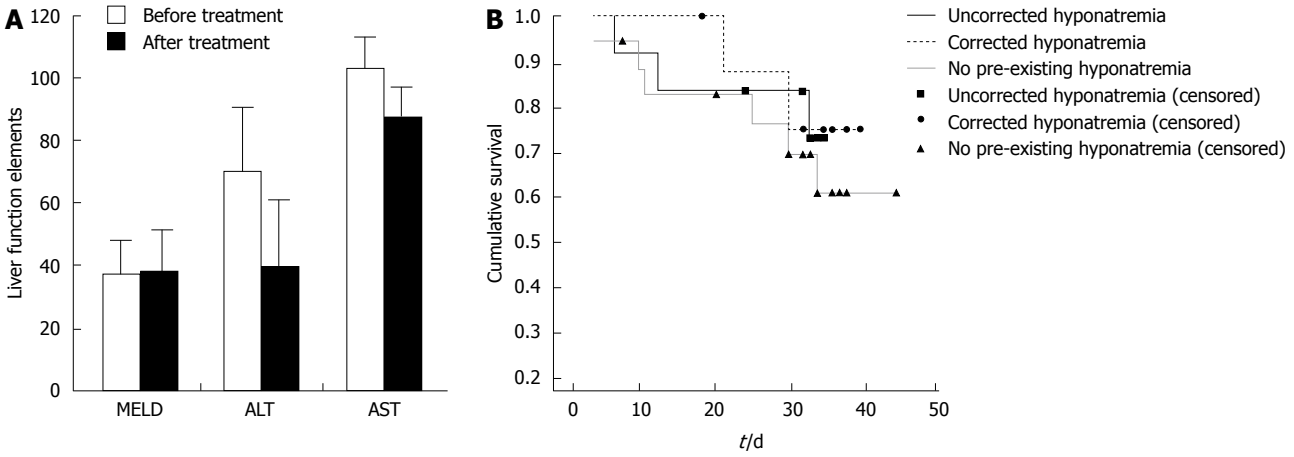

Figure 3 Tolvaptan does not affective liver function. A: Tolvaptan (15 mg/d for 5-14 d) does not affective liver function [model for end-stage liver disease (MELD) score, alanine aminotransferase (ALT) and aspartate aminotransferase (AST)] in patients with preexisting liver cirrhosis ($P > 0.05$); B: Kaplan-Meier analysis of the 1-mo survival of patients without hyponatremia, as well as with and without short-term correction of hyponatremia, did not show any significant difference between patient groups.

been explored previously in the subset of patients with refractory ascites. In the current study, the combination of tolvaptan with diuretics was effective in increasing urine output, decreasing abdominal circumference and reducing edema in patients with refractory ascites. Tolvaptan was effectively treated a substantial proportion of all patients, and 46.2% of the responders experienced significant improvement. Therefore, this study shows that the combination of tolvaptan with diuretics is more effective than standard options for treating refractory ascites in decompensated cirrhotic patients with or without further complications, such an hepatocellular carcinoma and/or hepatorenal syndrome.

A number of studies have demonstrated the efficacy of tolvaptan for treating hyponatremia[19-23]. In this study, tolvaptan significantly increased the serum sodium levels of patients with hyponatremia, while no significant difference was observed for patients lacking hyponatremia, supporting the efficacy of this drug in end-stage cirrhotic patients with refractory ascites and hyponatremia. A 1-mo follow-up assessment revealed that our treatment regimen corrected hyponatremia in 9 of 21 patients (42.9%). This observation is supported by reports from Berl *et al*[19]. While the relationship between the short-term correction of hyponatremia and survival was not statistically significant, this study had a small sample size. As a result, further studies are warranted to validate this finding. Further investigations are also warranted to definitively assess the clinical use of tolvaptan for treating refractory ascites in patients with decompensated liver cirrhosis.

In January 2013, the US FDA issued a warning for tolvaptan use because of the potential risks of liver injury that were identified during a clinical trial of tolvaptan to treat autosomal dominant polycystic kidney disease. The study found that 3 of 1445 cases treated with tolvaptan had significantly higher serum bilirubin and ALT. However, the clinical trial tolvaptan dose (120 mg/d for 3 years) was significantly higher, and the administration duration was longer than the recommended dosing strategy (15-60 mg/d for 7-30 d) for treating hyponatremia or ascites[24-26]. The United States, Japan and Europe are the main regions using tolvaptan in the clinic. Currently, there are only 311 cases of self-reported adverse events, and there are no reports of liver injury. Therefore, administering a lower dose of tolvaptan over a shorter treatment regimen does not affect liver function in patients with preexisting liver disease, such as liver cirrhosis.

In summary, the results of this observational study show that tolvaptan is effective to treat refractory ascites and/or edema in decompensated cirrhotic patients and is, therefore, a promising aquaretic agent.

ACKNOWLEDGMENTS

The authors would like to acknowledge Health Interactions Asia Pacific Pte Ltd for language polishing and the writing assistance of the Korea Otsuka Pharmaceutical Co., Ltd. We also thank Ms. Park YJ for her support in acting as a liaison for medical writing assistance.

COMMENTS

Background

Tolvaptan, a new oral, selective vasopressin V_2-receptor antagonist, is reported as an effective treatment for hyponatremia. Tolvaptan has a significant role in promoting aquaresis in patients with decompensated cirrhosis. However, the efficacy and safety of this drug for treating refractory ascites in cirrhotic patients and the effect(s) of tolvaptan use in patients with hepatorenal syndrome and/or hepatocellular carcinoma have not been studied previously.

Research frontiers

Numerous studies have reported the efficacy and safety of tolvaptan for the treatment of ascites and edema, and have demonstrated the efficacy of tolvaptan for treating hyponatremia in patients with decompensated cirrhosis.

Innovations and breakthroughs

This study shows that the combination of tolvaptan with diuretics is more effective than standard treatment approaches for refractory ascites in decompensated cirrhotic patients with or without further complications, such an hepatocellular carcinoma and/or hepatorenal syndrome.

Applications

Tolvaptan is a promising aquaretic agent that can be used to treat refractory ascites and/or edema in decompensated cirrhotic patients.

Terminology

Refractory ascites are defined as 1 wk of unresponsiveness to restrictions on salt intake and high-dose diuretics (more than 160 mg/d of furosemide and 200 mg/d of spironolactone) or recurrence occurring within 2 wk post-therapeutic paracentesis (removal of 3000-5000 mL of ascites fluid per treatment) and intermittent albumin infusion (10-20 g/d).

Peer review

This manuscript is the first reported open study showing that a short term (4-15 d) therapy with 15 mg/d of tolvaptan for refractory ascites in decompensated cirrhotic patients, as well as in patients with hepatorenal syndrome type 2 and hepatocellular carcinoma, is effective and safe. This is an interesting study.

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P- Reviewer: Guglielmi FW, Satapathy SK, Trapero-Marugan M, Wong GLH **S- Editor**: Gou SX **L- Editor**: Stewart G **E- Editor**: Wang CH

World Journal of Gastroenterology

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DOI: 10.3748/wjg.v20.i32.11406

World J Gastroenterol 2014 August 28; 20(32): 11406-11414
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

RANDOMIZED CONTROLLED TRIAL

Glutamine prevents oxidative stress in a model of mesenteric ischemia and reperfusion

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Supported by Grant from Pro-bolsas/PUCRS
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Received: January 17, 2014 Revised: March 14, 2014
Accepted: April 30, 2014
Published online: August 28, 2014

Abstract

AIM: To evaluate preventative effects of glutamine in an animal model of gut ischemia/reperfusion (I/R).

METHODS: Male Wistar rats were housed in a controlled environment and allowed access to food and water *ad libitum*. Twenty male Wistar rats were divided into four experimental groups: (1) control group (control) - rats underwent exploratory laparotomy; (2) control + glutamine group (control-GLU) - rats were subjected to laparotomy and treated intraperitoneally with glutamine 24 and 48 h prior to surgery; (3) I/R group - rats were subjected to occlusion of the superior mesenteric artery for 30 min followed by 15 min of reperfusion; and (4) ischemia/reperfusion + glutamine group (G + I/R) - rats were treated intraperitoneally with glutamine 24 and 48 h before I/R. Local and systemic injuries were determined by evaluating intestinal and lung segments for oxidative stress using lipid peroxidation and the activity of superoxide dismutase (SOD), interleukin-6 (IL-6) and nuclear factor kappa beta (NF-κB) after mesenteric I/R.

RESULTS: Lipid peroxidation of the membrane was increased in the animals subjected to I/R ($P < 0.05$). However, the group that received glutamine 24 and 48 h before the I/R procedure showed levels of lipid peroxidation similar to the control groups ($P < 0.05$). The activity of the antioxidant enzyme SOD was decreased in the gut of animals subjected to I/R when compared with the control group of animals not subjected to I/R ($P < 0.05$). However, the group that received glutamine 24 and 48 h before I/R showed similar SOD activity to both control groups not subjected to I/R ($P < 0.05$). The mean area of NF-κB staining for each of the control groups was similar. The I/R group showed the largest area of staining for NF-κB. The G + I/R group had the second highest amount of staining, but the mean value was much lower than that of the I/R group ($P < 0.05$). For IL-6, control and control-GLU groups showed similar areas of staining. The I/R group contained the largest area of IL-6 staining, followed by the G + I/R animals; however, this area was significantly lower than that of the group that underwent I/R without glutamine ($P < 0.05$).

CONCLUSION: These results demonstrate that pretreatment with glutamine prevents mucosal injury and improves gut and lung recovery after I/R injury in rats.

Key words: Ischemia-reperfusion; Glutamine; Lipid peroxidation; Superoxide dismutase; Nuclear factor-kappa beta; Interleukin 6

Core tip: Ischemia-reperfusion (I/R) leads to oxidative stress, with local and systemic consequences. Many enzymes and interleukins have been implicated in this process, among them interleukin-6 (IL-6) and nuclear factor kappa beta (NF-κB). The exact role of these enzymes is still not clear. Some substances, such as glutamine, have been studied as protective agents against oxidative stress. In an animal experimental model of intestinal I/R we have found that glutamine reduced lipid peroxidation, preserved superoxide dismutase activity, and decreased the expression of IL-6 and NF-κB in both lung and intestine, suggesting a protective role of this amino acid in the setting of intestinal I/R.

Zabot GP, Carvalhal GF, Marroni NP, Hartmann RM, da Silva VD, Fillmann HS. Glutamine prevents oxidative stress in a model of mesenteric ischemia and reperfusion. *World J Gastroenterol* 2014; 20(32): 11406-11414 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11406.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11406

INTRODUCTION

Ischemic conditions such as arterial occlusions, transplants, mesenteric ischemia and shock occur commonly in medical practice and affect a growing number of individuals of various ages, leading to high morbidity and mortality. However, unlike ischemic injuries, reperfusion injuries alter not only the affected areas but also produce systemic changes, so that the reestablishment of the blood flow to ischemic areas may result in damage to the entire body. The damage to remote organs is termed post-traumatic multiple organ failure (MOF)[1].

Gut ischemia usually results from occlusion of the celiac trunk and/or the superior mesenteric artery by thrombi or emboli and, more frequently, from non-occlusive processes, such as in the case of decreased mesenteric blood flow that occurs in heart failure and sepsis[2]. In the gut, ischemia followed by reperfusion frequently results in MOF, with the gut being the organ that triggers the injury process in distant organs. A systemic inflammatory reaction is initiated from pro-inflammatory substances released by the gut into the lymphatic circulation, with the inflammation mainly affecting lungs, liver and kidneys[3].

Although the details about the molecular mechanisms that determine injuries in ischemic events are not yet well defined, it is known that reactive oxygen species (ROS) play an important role in the pathogenesis of gut injury after an ischemia/reperfusion (I/R) event[4]. Parks and Granger reported that the tissue damage that occurs during reperfusion is greater than the injury that occurs during ischemia. Rupture of the mucosal barrier, bacterial translocation and activation of the inflammatory response, as well as acid-base balance and electrolyte disorders, are observed[5]. Superoxide and hydrogen peroxide are thought to be the main free radicals that contribute to I/R injury. Under normal conditions ROS are neutralized by endogenous antioxidant enzymes, but an excess of free radicals is observed during reperfusion, which results in oxidative stress[6]. Those free radicals originate when oxygen (O_2) is reintroduced into the ischemic tissue during reperfusion. Superoxide dismutase (SOD) is an antioxidant enzyme highly specific for superoxide elimination, thus reducing gastrointestinal lesions caused by I/R[7].

Nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-κB) is a transcription factor that plays a crucial role not only in normal states but also in the coordination of adaptive immune responses by regulating the expression of many cell mediators[8]. This factor, which was first described in 1986 by Sen and Baltimore[9], binds to specific kappa binding sites in the immunoglobulins of B cells. It is now well recognized that NF-κB is expressed in most cell types and that NF-κB consists of a dimer composed of members of the Relish (Rel) family. The NF-κB/Rel family contains five subunits, p50, p52, p65 (RelA), c-Rel and Rel-B. These subunits form homo- and heterodimers in several combinations. Generally, NF-κB is composed of two polypeptides, one of 50-kDa (p50) and one of *65-kDa* (p65). In homeostatic cells, NF-κB remains in the cytoplasm in its inactive form, associated with proteins that inhibit the kB site called kB inhibitors (IkB). Seven IkB isoforms have been described: IkBα, IkBβ, IkBγ, IkBε, Bcl-3, p100 and p105. NF-κB is activated by a variety of signals relevant to the etiology and pathophysiology of inflammation[8]. Intracellular and/or extracellular stimuli such as bacterial products (endotoxins, peptidoglycans), viruses and viral components, protozoa, cytokines (tumor necrosis factor-alpha (TNF-α), interleukins), free radicals and/or oxidants are needed to activate NF-κB[8]. In 2002, Haddad[10] suggested that NF-κB activation controls the oxidant/antioxidant balance.

Interleukin (IL)-6 and TNF-α levels are elevated in I/R as well as in MOF. Measurements of plasma levels of these endotoxins are important to assess the systemic effects of gut I/R. ROS promote oxidative stress as a result of the production of inflammatory cytokines, such as IL-6 and TNF-α, in addition to promoting the activation of neutrophils. IL-6 and TNF-α not only directly induce tissue damage but are also potent neutrophil activators. When sequestered in the intestinal tissue, these mediators and their enzymatic products promote increased micro vascular permeability, interstitial and perivascular edema, MOF and pulmonary edema[11].

The damage and loss of mucosal barrier integrity promotes bacterial translocation and the production of cytokines. The next stage is the transport of inflammatory mediators through the intestinal lymphatic system. The lung is the first exposed organ[12]. After resuscitation from hemorrhagic shock, lymph duct li-

gation prevents remote lung injury, the so-called "gut-lymph hypothesis"[13]. Lymphatic thoracic duct ligation prior to mesenteric I/R protects against lung injuries and modulates serum levels of endotoxins, D-lactate, diamine oxidase and cytokines. MOF causes acute lung injury (ALI) through the production of inflammatory mediators drained through the circulatory system. The gastrointestinal tract has the largest lymphatic system of the body. Mediators released by activated inflammatory cells during an acute event reach the interstitium, which is predominantly drained by lymphatics[13].

Several substances have been used for the treatment and/or prevention of experimental colitis. The experiments aimed to evaluate new drugs for the treatment of inflammatory processes or combinations of drugs to achieve better results[14]. Substances that inhibit or minimize the inflammatory process caused by aggressive agents, such as glutamine, have been used for prevention purposes. Glutamine is an uncharged, polar amino acid that is non-essential or occasionally essential, hydrophilic, and found on the surface of proteins where it interacts with water. Glutamine is the most abundant amino acid in peripheral blood[15]. This substance was initially used prophylactically in patients undergoing radiation therapy, leading to a reduction in the incidence and severity of actinic enteritis[16]. Glutamine also has a major role in the immune defense of the intestinal mucosal barrier due to its participation in the formation of immunoglobulins, especially IgA. Glutamine decreases the inflammatory effects of methotrexate-induced enterocolitis and reduces bacterial translocation in animals with abdominal sepsis[17]. This amino acid acts on macrophage activity, interfering with phagocytosis at inflammatory sites. In addition to the direct protective effects mentioned above, glutamine plays an important role in intestinal inflammatory processes by acting on ROS[14]. Glutamine is a multifunctional amino acid used for the synthesis of urea in the liver, renal aminogenesis, gluconeogenesis, and as the main respiratory fuel for many cells. Low glutamine concentrations are found during catabolic stress and are associated with susceptibility to infections. Glutamine is not only an important energy source for mitochondria but is also a precursor of the brain neurotransmitter glutamate, which then participates in the synthesis of the antioxidant glutathione[15]. Glutamine is thus vital in the regulation of the intracellular oxidative balance[16]. Glutamine has been used as a nutritional supplement in severely debilitated patients to reduce the deleterious effects of oxidative stress[18]. It has been shown that preventing oxidative stress in patients with severe conditions or multiple traumas or undergoing major surgery is useful as a treatment adjunct. In this setting, antioxidant therapy improves patient prognosis and decreases the overall rate of complications[19].

Clinical observations have shown that patients receiving dietary glutamine supplementation had a better tolerance to colitis resulting from radiation therapy for prostate and cervical neoplasms[16]. The same substance was then used in patients with Crohn's disease (granulomatous enterocolitis) and ulcerative rectocolitis. A clinical improvement was observed in these patients, namely decreased diarrhea, increased fistulae healing rates and decreased use of medications. Because of the importance of active oxygen species in the genesis of colitis, the relationship between oxidative stress and the supposed beneficial clinical effect of glutamine in colitis has become a subject of research. The mechanism by which glutamine exerts beneficial effects appears to be associated with the biosynthesis of glutathione, which causes a consequent reduction in lipid peroxidation of the intestinal membrane during mesenteric I/R[20].

The aim of our study was to investigate the effects of glutamine treatment in an animal model of mesenteric I/R analyzing parameters such as lipid peroxidation, SOD activity, and immunohistochemical expression of IL-6 and NF-κB.

MATERIALS AND METHODS

Ethics

Animal care was in compliance with the normative resolution 04/97 of the Research and Ethics Committee of the Health Research Group and Graduate Teaching Hospital of Porto Alegre (Hospital de Clinicas de Porto Alegre-HCPA)[21].

Animals

Male Wistar rats [250-300 g; State Foundation for Production and Health Research (Fundação Estadual de Produção e Pesquisa em Saúde-FEPPS)] were housed in a controlled environment and allowed access to food and water *ad libitum*.

Surgical procedures

After trichotomy, rats were anesthetized with ketamine and xylazine solution [45 mg/kg intraperitoneally (*ip*)]. After midline laparotomy, the celiac and superior mesenteric arteries were isolated near their aortic origins. During this procedure, the intestinal tract was placed between gauze pads soaked with warm 0.9% NaCl solution. The superior mesenteric artery and the celiac trunk were clamped, resulting in total occlusion of these arteries for 30 min to induce splanchnic artery occlusion injury. After occlusion, the clamps were removed, and after 15 min of reperfusion, intestinal segments (10 cm) and pieces of the lung were removed for histological examination and biochemical studies.

Experimental groups

Rats were randomly allocated into the following groups: (1) ischemia/reperfusion (I/R): rats were subjected to splanchnic artery occlusion injury (30 min) followed by reperfusion (15 min) (n = 5); (2) ischemia/reperfusion + glutamine group (G + I/R): identical to the ischemia/reperfusion group but were treated with glutamine (25 mg/kg *ip*) 24 and 48 h before I/R (n = 5); (3) control

group (control): rats were subjected to identical surgical procedures as the above groups, except the blood vessels were not occluded and the rats were maintained under anesthesia for the duration of the experiment (n = 5); and (4) control + glutamine group (control-GLU): identical to the Control group except for the administration of glutamine (25 mg/kg *ip*) 24 and 48 h before identical surgical procedures (n = 5). The glutamine treatment dose of 25mg/kg *ip* was chosen based on previous studies[22].

Assessment of lipid peroxidation

Thiobarbituric acid reactive substances: Tissue samples were placed in test tubes; solutions were added in the following order: 0.75 mL of 10% trichloroacetic acid (TCA), 0.25 mL of homogenate, 0.5 mL of 0.67% thiobarbituric acid (TBA), and 0.25 mL of distilled water.

Thiobarbituric acid reactive substances (TBARS) consists of heating the homogenate with thiobarbituric acid and measuring the consequent formation of a colored product in a spectrophotometer at 535 nm. The coloration is due to the presence of malondialdehyde and other substances from biological lipid peroxidation[23].

SOD activity

SOD was measured according to Misra and Fridovich. The rate of auto-oxidation of epinephrine, which is inhibited by SOD, is measured in the presence of progressively increasing doses of SOD with a spectrophotometer at 560 nm. The amount of enzyme that inhibits auto-oxidation of epinephrine at 50% of the maximum dose is defined as 1 U SOD[24].

Evaluation of NF-κB and IL-6

To prepare slides for subsequent immunohistochemical analysis, tissue was sectioned at 3-μm thickness using a microtome (Leica SM 2000R, Germany). The sections were placed on slides pretreated with HistoGrip (Zymed, United States) and incubated in an oven at 60 ℃ for 24 h.

Sections were deparaffinized by incubating in xylene three times for 10 min, followed by rehydration of the sections using decreasing concentrations of ethanol. Antigen exposure was performed using the pTLINK platform (DAKO) for 40 min at 98 ℃ with the Envision Flex antigen retrieval solution, high pH (DAKO). The slides were then immediately washed in phosphate-buffered saline (PBS), pH 7.2. The blocking of endogenous peroxidases was performed with two 15-min incubations in a 3% solution of H_2O_2 in methyl alcohol, which were followed by three washes with PBS, pH 7.2. Non-specific binding was blocked using the commercial solution Serum-Free Protein Block (Dako, United States) for 30 min at room temperature.

The sections were incubated using the immunostaining Sequenza station (Thermo Shandon, United States) overnight at 2 ℃ and 6 ℃ and with the following primary antibodies diluted in Antibody Diluent with Background Reducing Components (Dako, United States): anti-NF-κB (Santa Cruz Biotechnology, United States) at 1:100 and anti-IL-6 (Santa Cruz Biotechnology, United States) at 1:100. After incubation with the primary antibody, sections were washed three times in PBS, pH 7.2. To amplify the antigen-antibody reaction, the Advance system HRP was used for IL-6 (Dako, United States) according to the manufacturer's recommendations, and for NF-κB, goat anti-rabbit IgG-HRP secondary antibody was used at 1:300 in PBS for 30 min at room temperature. Next, the slides were washed with PBS and incubated with diaminobenzidine (Dako Liquid DAB Substrate Chromogen System, United States) for 5 min. After washing with distilled water, slides were counterstained with Harris hematoxylin for 1 min, washed with water until complete removal of the dye and incubated in a 37 mmol/L ammonia solution for 15 s. Finally, the slides were dehydrated in absolute ethanol (four incubations of 2 min) and two treatments with xylene for 5 min. The slides were mounted with Entellan synthetic medium (Merck, Germany)[25].

Analysis of digital images

We used a digital analysis system composed of a Zeiss Axioskop 40 microscope (Oberkochen, Germany) with Neofluar lenses connected by a Roper Scientific video camera (Media Cybernetics, Rockville, United States) to a computer with an Image Capture Pro kit (Media Cybernetics, Rockville, MD, United States) capture card. Image Pro Plus version 4.5 (Media Cybernetics, Rockville, United States) was used to analyze digital images. The images were captured in TIFF (True Image File Format) format without compression by the same examiner with a light intensity pattern for all photos. Images were captured of at least fifteen random, non overlapping fields for each histological slide at 200 × magnification (44 pixel = 1 μm). The hot spot method was used to select fields on slides with focal positivity for the markers. Color selection was performed interactively by three trained observers and was then applied to all samples by the automated digital image analysis system. The initial area considered was 0.01 cm.

Statistical analysis

Quantitative data were initially described by mean and standard deviation. To compare groups, we used analysis of variance. For categorical data, we used scores and comparisons based on Fisher's exact test.

Analysis of variance with robust standard errors (Welch) was used to verify NF-κB and IL-6 results between groups.

The significance level for the experiments was $P < 0.05$. Data were analyzed with SPSS version 21.0.

RESULTS

Evaluation of oxidative stress by analysis of lipid peroxidation

Lipid peroxidation of the membrane was increased in both the gut and the lung in the animals subjected to I/R ($P < 0.05$). However, the group that received glutamine

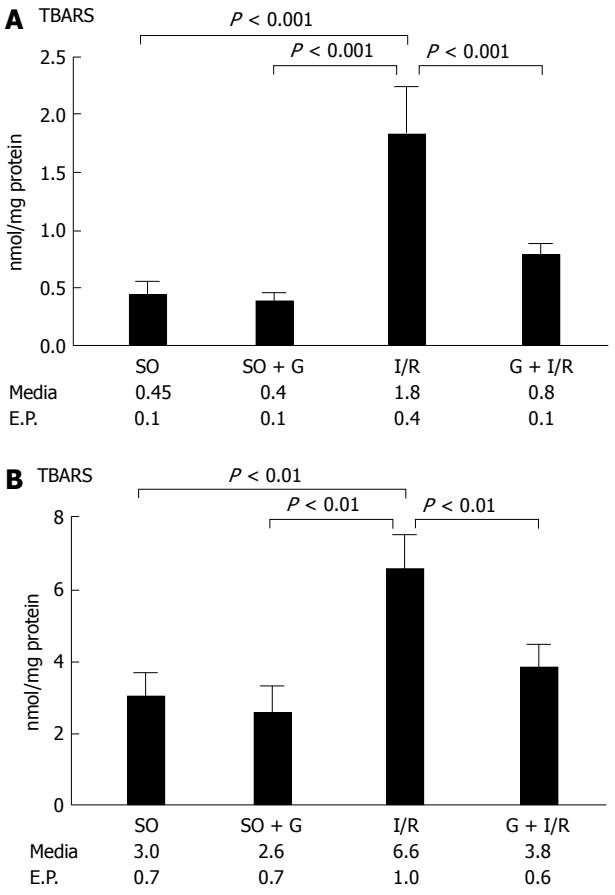

Figure 1 Lipid peroxidation (thiobarbituric acid reactive substances). A: In the gut; B: In the lungs. TBARS: Thiobarbituric acid reactive substances; SO: Control group means. I/R: Ischemia/reperfusion.

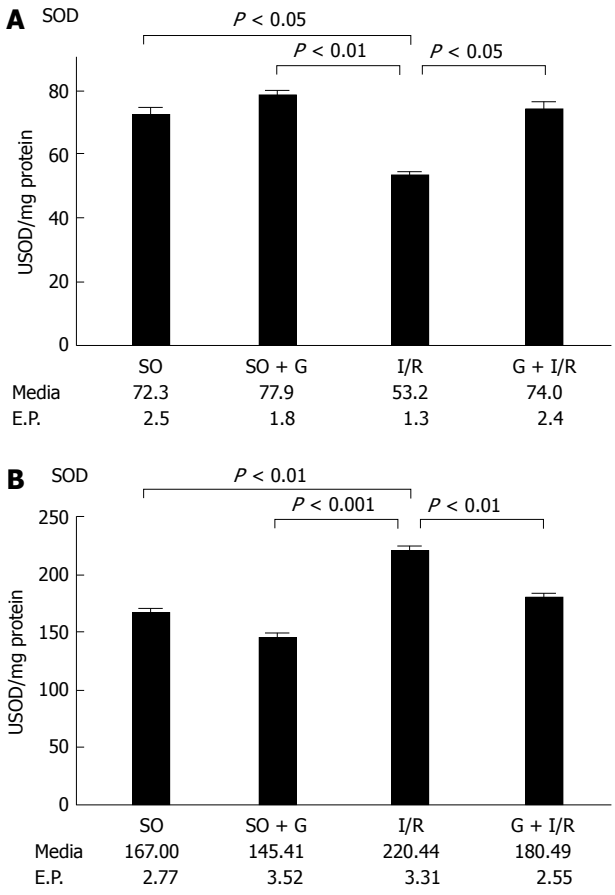

Figure 2 Levels of superoxide dismutase. A: In the gut; B: In the lungs. SOD: Superoxide dismutase; SO: Control group means. I/R: Ischemia/reperfusion.

24 and 48 h before the I/R procedure showed levels of lipid peroxidation similar to the control groups (animals not subjected to I/R and also the group receiving glutamine without I/R) that were significantly different from animals that only received I/R ($P < 0.05$). These results are shown in Figure 1.

SOD activity

Figure 2 shows that the activity of the antioxidant enzyme SOD was decreased in the gut of animals subjected to I/R. These findings were statistically significant ($P < 0.05$) when compared with the control group of animals not subjected to I/R. However, the group that received glutamine 24 and 48 h before I/R showed similar SOD activity to both control groups not subjected to I/R. There was a significant difference between the group of animals subjected to I/R and the group that received glutamine before I/R, suggesting that glutamine is a protective factor for mesenteric I/R.

NF-κB transcription factor

We calculated the mean area of NF-κB staining for each of the groups. As shown in Figures 3 and 4, the control and control-GLU groups presented similar mean areas. The I/R group showed the largest area of staining. The G + I/R group had the second highest amount of staining, but the mean value was much lower than that of the I/R group. The same differences were observed among groups in the large intestine and the lung. These findings were statistically significant ($P < 0.05$).

Evaluation of IL-6

Images of IL-6 staining were analyzed in the same method as those stained for NF-κB. As shown in Figures 5 and 6, the control and control-GLU groups showed similar areas of staining. The I/R group contained the largest area of staining, followed by the G + I/R animals; however, this area was significantly lower than that of the group that underwent I/R without glutamine ($P < 0.05$).

DISCUSSION

As glutamine is glutathione precursor, and glutathione is the main non-enzymatic cellular antioxidant, is vital in the regulation of the intracellular oxidative balance[26].

This study demonstrates that glutamine treatment exerts important protective effects against splanchnic artery occlusion injury in a murine model. Our data provide evidence that glutamine attenuates: (1) the lipid peroxidation of gut mucosa; (2) the decrease in SOD activity; (3) the

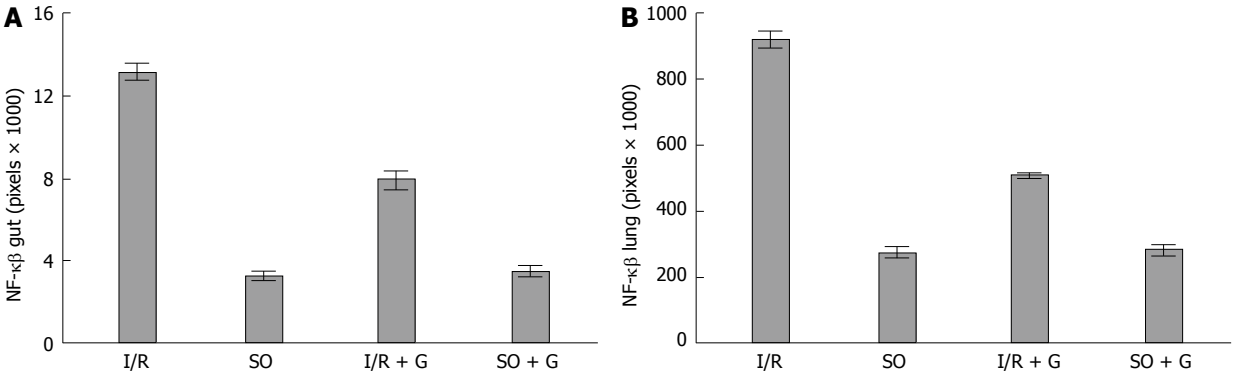

Figure 3 Immunohistochemical expression of nuclear factor kappa beta. A: In the gut; B: In the lungs. SO: Control group means; I/R: Ischemia/reperfusion; NF-κB: Nuclear factor kappa beta.

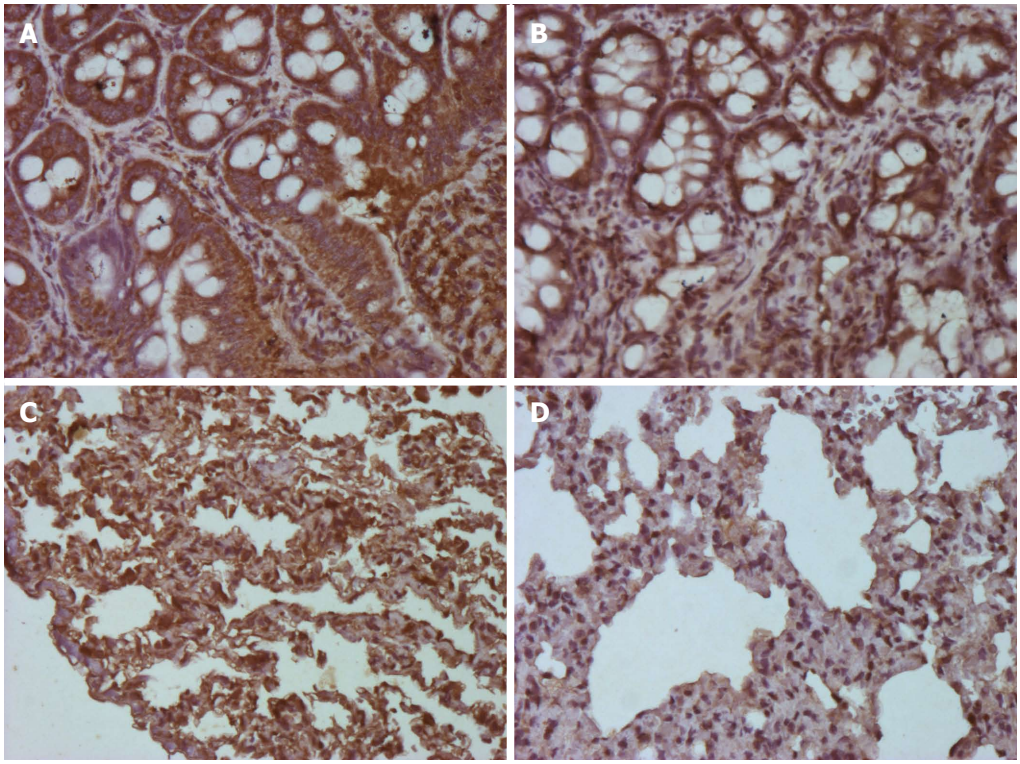

Figure 4 Digital images of the immunohistochemical expression of nuclear factor kappa beta (photomicrography, × 200). A: Ischemia/reperfusion (I/R) gut; B: G + I/R gut; C: I/R lung; D: G + I/R lung.

increases in NF-κB expression; and (4) IL-6 expression that occur after I/R.

In our study, the amount of lipid peroxidation was greater in the group of animals subjected to I/R. The addition of glutamine significantly decreased lipid peroxidation compared with animals that underwent I/R without glutamine treatment. Other authors, such as Mondello *et al*[27] and He *et al*[13] observed similar beneficial results of glutamine for I/R with different methodologies. Mondello *et al*[27] have induced intestinal ischemia in rats by clamping the superior mesenteric artery and the celiac trunk for 30 min, then releasing it and promoting reperfusion during 1 h. Glutamine was administered 15 min before reperfusion at the dose of 1.5 mg/kg, *iv* Their findings showed a reduction in: (1) the infiltration of neutrophils in the ileum; (2) the formation of the pro-inflammatory cytokines; (3) the expression of the adhesion molecules ICAM-1 and P-selectin; (4) the IKB-α degradation and the nuclear translocation of NF-κB; and (5) the nitrotyrosine formation and PARP activation. He *et al*[13] utilized a rat model of I/R, but administering glutamine enterically before and after a 60 min ischemia; additionally, in one subgroup the lymphatic mesenteric duct was also ligated before the production of intestinal ischemia. They concluded that both the enteral administration of glutamine and the ligature of the lymphatic mesenteric duct prevented intestinal permeability, attenuating systemic inflammatory reactions and ALI. In contrast, Fukatsu *et al*[28] have shown that in a murine model of gut I/R, an *iv* glutamine was detrimental in terms of

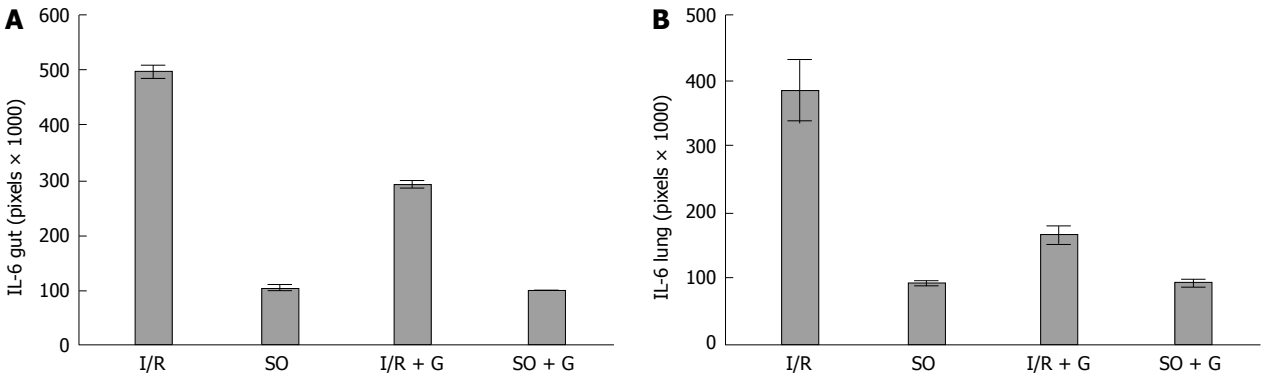

Figure 5 Immunohistochemical expression of interleukin-6. A: In the gut; B: In the lungs. SO: Control group means; I/R: Ischemia/reperfusion; IL: Interleukin.

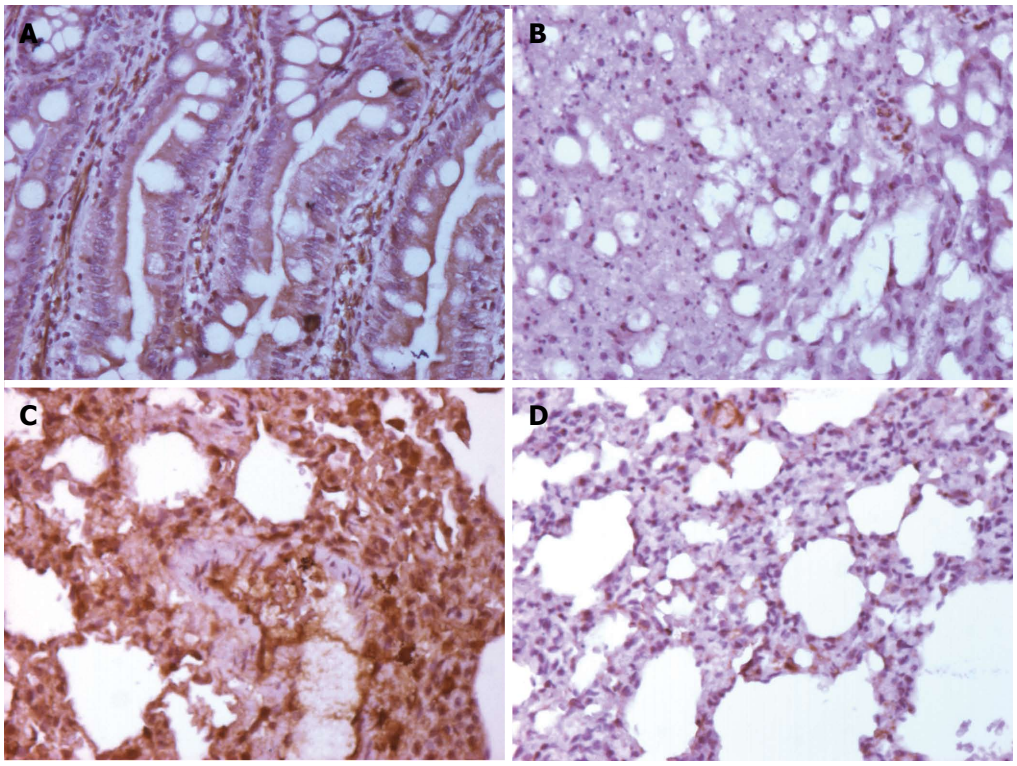

Figure 6 Digital images of the immunohistochemical expression of interleukin-6 (photomicrography, × 200). A: Ischemia/reperfusion (I/R) gut; B: G + I/R gut; C: I/R lung; D: G + I/R lung.

survival and organ injury due to the increased priming of circulating myeloid cells.

In our study, SOD activity was decreased in animals submitted to I/R. In the Control and Control-GLU groups, the decrease in SOD activity was much lower and similar between the two groups. The addition of glutamine to animals submitted to I/R produced a decrease that was not as significant but that was lower than that found in the I/R only group. The first authors to describe the role of SOD in oxidative stress were Misra and Fridovich[24]. In their pivotal study, SOD was prepared from bovine erythrocytes, being able to inhibit the autoxidation of epinephrine at a pH 10.2. Recently, Salman *et al*[29] administered glutamine by gavage to Sprague-Dawley rats, at a dose of 1 g/kg for 10 d prior to intestinal I/R, studying tissue damage in the intestines and lungs. These authors measured the intestinal and pulmonary levels of SOD, in addition to serum levels of TNF-α and IL-6, concluding that pre-treatment with a bolus dose of enteral glutamine was able to minimize the extent of ALI in rats.

Tissue I/R activates families of protein kinases that converge on specific transcription factors (protein activator-1 (PA-1) and NF-κB) that regulate the expression of pro-inflammatory genes. In our study, the activity of NF-κB was higher in both the intestines and the lungs in the group subjected to I/R. However, in the group of animals that received prophylactic glutamine *ip* 24 and 48 h before I/R, the levels of NF-κB were lower. This difference between groups was statistically significant. Sen and Baltimore[9] published the first report on NF-κB, suggesting its important role in cellular inflammatory response to injury. However, the exact role of this transcription factor re-

mains controversial. According to Haddad[10], NF-κB appears to perform an important function in the generation and resolution of intestinal I/R lesions, as a transcription factor that is directly influenced by reactive species and pro-inflammatory signs. Bowie *et al*[30], in a review article about oxidative stress and Nf-kB activation, determined that in most cases, the role of oxidative stress in NF-κB activation is at best facilitatory rather than causal, if a there exists a role at all. Ypsilantis *et al*[31] tested the hypothesis that the action of 2-mercaptoethane-sulfonate (mesna) is mediated by the inhibition of NF-κB, studying the oxidative stress on a rat model of I/R, analyzing glutathione, malondialdehyde concentration, SOD and NF-κB. These authors concluded that prophylaxis with mesna prevents oxidative stress induced by I/R in the intestine via inhibition of NF-κB activation.

ROS-mediated oxidative injury as a consequence of increased production of inflammatory cytokines such as IL-6 and TNF-α and the neutrophil activation play critical roles in the pathogenesis of I/R. IL-6 and TNF-α not only directly induce tissue damage but are also potent activators of neutrophils. The neutrophils and their enzymatic products cause increased microvascular permeability, perivascular and interstitial edema, and even promote distant organ injury such as pulmonary edema when sequestrated in intestinal tissue. Cuzzocrea *et al*[11] studied the inflammatory process secondary to I/R in a knockout mice model, verifying by immunohistochemistry that IL-6 plays an important role in I/R injury, suggesting that the inhibition of IL-6 may actually represent a novel and possible strategy in the prevention of I/R injuries.

In our study, similarly to NF-κB, the immunohistochemical expression of IL-6 was found to be high in animals that underwent I/R in both the intestines and the lungs. The control and control-GLU groups showed similar results for IL-6, with observed levels well below those of the I/R group. However, the group that received a potentially protective factor, glutamine, before I/R showed a higher expression of IL-6 than the control and control-GLU groups but at levels that were statistically inferior to the I/R group.

In conclusion, this study demonstrates that *ip* administration of glutamine at a dose of 25 mg/kg 24 and 48 h before animals are subjected to 30 min of mesenteric ischemia and 15 min of reperfusion effectively protected against lipid peroxidation and preserved SOD activity. The activity of NF-κB and IL-6 were also reduced upon *ip* administration of glutamine at 24 and 48 h prior to I/R in rats. This adds to previously published data on glutamine as a protective factor in mesenteric I/R states in rats. Further studies are necessary to test the role of glutamine as a potential protective agent against I/R lesions in humans.

COMMENTS

Background

Ischemia-reperfusion (I/R) leads to oxidative stress, with local and systemic consequences. Many enzymes and interleukins have been implicated in this process, among them interleukin-6 (IL-6) and nuclear factor kappa beta (NF-κB). The exact role of these enzymes is still not clear. Substances that inhibit or minimize the inflammatory process caused by aggressive agents, such as glutamine, have been used for prevention purposes.

Research frontiers

Glutamine is the most abundant amino acid in peripheral blood. That amino acid acts on macrophage activity, interfering with phagocytosis at inflammatory sites. Plays an important role in intestinal inflammatory processes by acting on reactive oxygen species (ROS). Glutamine is a multifunctional amino acid used for the synthesis of urea in the liver, renal *aminogenesis*, gluconeogenesis, and as the main respiratory fuel for many cells.

Innovations and breakthroughs

This substance was initially used prophylactically in patients undergoing radiation therapy, leading to a reduction in the incidence and severity of actinic enteritis. It is thus vital in the regulation of the intracellular oxidative balance. Glutamine has been used as a nutritional supplement in severely debilitated patients to reduce the deleterious effects of oxidative stress. The present study demonstrated that the pretreatment with glutamine prevents mucosal injury and improves gut and lung recovery after I/R injury in a rat model.

Applications

The study results suggest that the glutamine protected against lipid peroxidation and preserved superoxide dismutase (SOD) activity. The activity of NF-κB and IL-6 were also reduced upon *ip* administration of glutamine at 24 and 48 h prior to I/R in rats. This adds to previously published data on glutamine as a protective factor in mesenteric I/R states in rats.

Terminology

Ischemia/reperfusion (I/R): gut ischemia usually results from occlusion of the celiac trunk and/or the superior mesenteric artery by thrombi or emboli and, more frequently, from non-occlusive processes, such as in the case of decreased mesenteric blood flow that occurs in heart failure and sepsis. In the gut, ischemia followed by reperfusion frequently results in multiple organ failure (MOF), with the gut being the organ that triggers the injury process in distant organs; SOD: is an antioxidant enzyme highly specific for superoxide elimination, thus reducing gastrointestinal lesions caused by I/R; NF-κB: nuclear factor of kappa light polypeptide gene enhancer in B-cells is a transcription factor that plays a crucial role not only in normal states but also in the coordination of adaptive immune responses by regulating the expression of many cell mediators; IL-6: levels are elevated in I/R as well as in MOF; Glutamine: is a polar amino acid that is *non-essential* or occasionally essential, hydrophilic, and found on the surface of proteins where it interacts with water.

Peer review

This is an interesting article studying how the pretreatment with glutamine prevents mucosal injury and improves gut and lung recovery after I/R injury in a rat model. The manuscript includes six clear figures. This research is easy to follow and finds some valuable information for scientific community interested in both glutamine and ischemia/reperfusion, as well as in oxidative damage and ROS. To date, this is the first investigation to study glutamine effect on NF-κB and IL-6, as well as in SOD and TBARS in a model of mesenteric ischemia/reperfusion.

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P- Reviewer: Matés JM **S- Editor**: Ma YJ **L- Editor**: A
E- Editor: Wang CH

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DOI: 10.3748/wjg.v20.i32.11415

World J Gastroenterol 2014 August 28; 20(32): 11415-11421
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

RANDOMIZED CLINICAL TRIAL

Furazolidone-based triple and quadruple eradication therapy for *Helicobacter pylori* infection

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Supported by The National Science and Technology Major Projects for "Major New Drugs Innovation and Development" of China, No. 2011ZX09302-007-03; Jiangxi Province Talent 555 Project, and Jiangxi Society of Gastroenterology
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Received: November 14, 2013 Revised: March 16, 2014
Accepted: May 29, 2014
Published online: August 28, 2014

Abstract

AIM: To evaluate the efficacy of furazolidone-based triple and quadruple therapy in eradicating *Helicobacter pylori* (*H. pylori*) in a multi-center randomized controlled trial.

METHODS: A total of 720 *H. pylori* positive patients with duodenal ulcer disease were enrolled at 10 different hospitals in Jiangxi province in China. The patients were randomly assigned to four treatment groups as follows: patients in Groups 1 and 3 received rabeprazole (10 mg), amoxicillin (1000 mg) and furazolidone (100 mg) twice daily for 7 and 10 d, respectively; patients in Groups 2 and 4 received rabeprazole (10 mg), bismuth (220 mg), amoxicillin (1000 mg) and furazolidone (100 mg) twice daily for 7 and 10 d, respectively. The primary outcome measure was *H. pylori* eradication rate 4 wk after treatment by intention-to-treat and per protocol analysis, while the secondary outcome measures were symptom and sign changes at the end of treatment and 4 wk after the end of treatment, as well as the proportion of patients who developed adverse events.

RESULTS: The demographic data of the four groups were not significantly different. Overall, 666 patients completed the scheme and were re-assessed with the ^{13}C-urea breath test. The intention-to-treat analysis of the *H. pylori* eradication rates in Groups 1, 2, 3 and 4 were 74.44%, 82.78%, 78.89% and 86.11%, respectively. The *H. pylori* eradication rate in Group 4 was significantly higher than that in Group 1. According to

the per protocol analysis, the *H. pylori* eradication rates in Groups 1, 2, 3 and 4 were 81.21%, 89.22%, 85.54% and 92.26%, respectively. The *H. pylori* eradication rate in Group 4 was significantly higher than that in Group 1. The number of adverse events was 15 (8.3%), 16 (8.9%), 15 (8.3%) and 17 (9.4%) in Groups 1, 2, 3 and 4, respectively, including dizziness, vomiting, diarrhea, nausea, skin rash, itchy skin, and malaise. The symptoms were relieved without special treatment in all of the patients.

CONCLUSION: Both 7- and 10-d quadruple furazolidone-based therapies achieve satisfactory *H. pylori* eradication rates.

Key words: *Helicobacter pylori* infection; Furazolidone; Treatment; Eradication

Core tip: This is a large sample, multi-center reseach to evaluate the effects of furazolidone based regimens in *Helicobacter pylori* (*H. pylori*) eradication. In this study, the efficacy of furazolidone-based triple and quadruple therapies was investigated in patients with *H. pylori* positive duodenal ulcers. The present study found that both 7- and 10-d quadruple furazolidone-based therapies achieve satisfactory *H. pylori* eradication rates, which are recommended as an alternative treatment for *H. pylori* eradication.

Xie Y, Zhu Y, Zhou H, Lu ZF, Yang Z, Shu X, Guo XB, Fan HZ, Tang JH, Zeng XP, Wen JB, Li XQ, He XX, Ma JH, Liu DS, Huang CB, Xu NJ, Wang NR, Lu NH. Furazolidone-based triple and quadruple eradication therapy for *Helicobacter pylori* infection. *World J Gastroenterol* 2014; 20(32): 11415-11421 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11415.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11415

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative, microaerophilic bacterium associated with chronic gastritis and peptic ulcers and is linked to the development of gastric carcinoma and mucosa associated lymphoid tissue (MALT) tumors. The eradication rate of *H. pylori* should be higher than 90%[1]. The Maastricht Ⅳ Consensus[2] and the Fourth Chinese National Consensus Report[3] recommended standard triple and quadruple therapy with bismuth and a *H. pylori* eradication rate higher than 80% for the management of *H. pylori* infection. However, in recent years, the widespread use of antibiotics has led to the development of drug resistance to antibiotics used in the treatment of *H. pylori* infection. The eradication rate of *H. pylori* infection has fallen to less than 80% with standard triple therapy [proton pump inhibitor (PPI), clarithromycin and amoxicillin as a first-line regimen][1].

Furazolidone is a traditional antimicrobial agent and has antibacterial effects on both Gram-positive and -negative bacteria by interfering with bacterial oxidoreductase, causing metabolic disorders and death of the bacteria. The metabolites of nitrofuran drugs may have some carcinogenic effects in rodents; however, furazolidone has been extensively used to treat humans[4-6]. In addition, *H. pylori* has a low resistance rate to furazolidone[7]. Regimens that include furazolidone treatment achieve better eradication rates than clarithromycin-based regimens[8-10]. A multi-center trial in China showed that the eradication rates of three 7-d furazolidone-based triple therapies containing omeprazole, furazolidone and clarithromycin, or omeprazole, furazolidone and amoxicillin, or colloidal bismuth subcitrate, furazolidone and clarithromycin were 69.2%, 86.6% and 80.4%, respectively[8]. Cheng *et al*[11] used 7- and 14-d quadruple regimens that contained rabeprazole, bismuth potassium citrate, amoxicillin and furazolidone, and achieved eradication rates of 82% and 89%, respectively, indicating that furazolidone-based regimens are effective in *H. pylori* eradication. However, there is a lack of large sample, multi-center studies in patients with antibiotic resistance. In this study, the efficacy of furazolidone-based triple and quadruple therapies was investigated in patients with *H. pylori* positive duodenal ulcers (DU).

MATERIALS AND METHODS

Subjects

This multi-center randomized controlled study was conducted during January 2010 to June 2011 at 10 hospitals of the Jiangxi province in China (The First Affiliated Hospital of Nanchang University, Jiangxi Provincial People's Hospital, The People's Hospital of Yichun City, Ganzhou People's Hospital, The Third Hospital of Nanchang, Pingxiang People's Hospital, Fengcheng People's Hospital, The First Affiliated Hospital of Gannan Medical College, Yingtan City People's Hospital, The Fourth Affiliated Hospital of Nanchang University). The protocol of this prospective study was approved by the ethics committee of Nanchang University. All patients that underwent a gastroscopy for gastrointestinal symptoms and an active DU with *H. pylori* infection were enrolled after informed consent. In total, 720 patients were included in this study. Inclusion criteria for patients were as follows: (1) an active DU having a diameter of 0.5 to 1.0 cm without complications, including bleeding and perforation; (2) aged 16 to 70 years, both female and male; (3) did not receive *H. pylori* eradication therapy prior to enrollment; (4) positive rapid urease test of ++ or above; and (5) signed informed consent.

Exclusion criteria were as follows: (1) patients who were treated with antibiotics, bismuth agent, H_2 receptor antagonist (H_2RA), PPI or a mucosa protective agent two weeks before enrollment; (2) patients with gastric ulcers and esophageal ulcers; (3) patients who were pregnant or

lactating; (4) patients with other serious diseases, such as severe liver, heart and kidney disease, cancer and disorders caused by alcohol abuse; (5) known allergy to drugs being prescribed; (6) patients who participated in other drug research during the 3 mo prior to enrollment; and (7) patients who could not correctly express their complaints, such as those who had a mental illness, severe nervous functional disorders, or could not cooperate with researchers.

Criteria for terminating the study were as follows: (1) deterioration or severe complications; (2) serious adverse events that could not be tolerated during treatment; (3) other diseases that interfered with observation during treatment; (4) lost to follow-up; and (5) pregnancy during treatment.

Study drugs

The drugs used in this study, as well as the manufacturer, were as follows: rabeprazole (Rui Bote), Jiangsu HanSoh Pharmaceutical Group; amoxicillin (Imacil), the United Laboratories International Holding Limited; colloidal bismuth subcitrate (bismuth potassium citrate granules), Livzon Pharmaceutical Group; and furazolidone, Jinling Pharmaceutical Co., Ltd. Limin Pharmaceutical Factory.

Treatment regimens

The patients were randomly divided into the following four groups: Group 1, treated with rabeprazole (10 mg), amoxicillin (1000 mg) and furazolidone (100 mg) twice daily (*bid*) for 7 d; Group 2, treated with rabeprazole (10 mg), bismuth (220 mg), amoxicillin (1000 mg) and furazolidone (100 mg) *bid* for 7 d; Group 3, treated with rabeprazole (10 mg), amoxicillin (1000 mg) and furazolidone (100 mg) *bid* for 10 d; and Group 4, treated with rabeprazole (10 mg), bismuth (220 mg), amoxicillin (1000 mg) and furazolidone (100 mg) *bid* for 10 d. After the abovementioned treatments, every patient received ranitidine (150 mg) for 14 d (Groups 1 and 2) or 11 d (Groups 3 and 4).

Assessments

The diagnosis of *H. pylori* infection: A positive endoscopic rapid urease test indicated *H. pylori* infection[2,3].

The confirmation of *H. pylori* eradication: *H. pylori* eradication was confirmed when a ^{13}C-urea expiratory test was negative 4 wk after the completion of the study treatment[2,3].

Evaluation of symptoms and adverse events: Case record forms (CRF) of the patients were completed. The patients' symptom history was recorded and a physical examination was completed before treatment, 7 or 10 d after treatment, and 4 wk after completing the study treatment. Symptoms and signs, medications used other than those prescribed in the study, returned unused study drugs, adverse events and other information were carefully recorded throughout the study.

Evaluation of clinical symptom severity and treatment efficacy: The severity of upper gastrointestinal symptoms, including pain, burning sensation, acid regurgitation, nausea and vomiting, belching and abdominal distension were observed and rated, respectively. The symptom scores were as follows: 0, no symptoms; 1, light symptoms that did not affect daily life and/or work; 2, symptoms that affected daily life and/or work; and 3, serious symptoms that affected daily life and work, and medications were needed.

The improvement rate (IR) of clinical symptoms was calculated with the following formula: IR (%) = [(clinical symptom total points before treatment - the clinical symptom total points after treatment)/clinical symptom totals point before treatment] × 100%. The therapeutic efficacies of the triple and quadruple therapies were categorized into four groups: (1) very effective, reduction of all symptom scores to 0 or a reduction by 75%; (2) effective, reduction of symptom scores between $\geqslant$ 50% and $<$ 75%; (3) slightly effective, reduction of symptom scores between $\geqslant$ 25% and $<$ 50%; and (4) ineffective, reduction of symptom scores by $<$ 25% or increased symptom scores.

The main outcome measures

The primary outcome measure was *H. pylori* eradication rate 4 wk after treatment, while the secondary outcome measures were symptom and sign changes at the end of treatment and 4 wk after the end of treatment, as well as the proportion of patients who developed adverse events.

An intention-to-treat (ITT) analysis was performed, and all the patients who took at least one dose of a study drug were enrolled for analyzing the curative efficacy and adverse events. The last observational data were used as the final result for patients who failed to observe all treatment requirements.

A per-protocol (PP) analysis was performed on the data of patients who complied well with the protocol, did not take illicit drugs during the study period and completed the CRF form.

Statistical analysis

All the statistical analyses were performed with SPSS 16.0. The single factor analysis of variance was used for measurement data, the χ^2 test was used for unordered categorical data, and the rank-sum test (Kruskal-Wallis) was used for ordinal categorical data. All the statistical tests were two-tailed, and a P value of $<$ 0.05 was considered statistically significant.

RESULTS

Demography

A total of 720 patients were randomized and included in this study. The demographic data of the four groups were not significantly different (P = 0.337) (Table 1). Fifty-four patients were excluded in the follow-up period (36 lost to follow-up and 18 discontinued), and thus 666 patients

Table 1 The age and gender of the four groups of patients *n* (%)

		Group 1 (*n* = 180)	Group 2 (*n* = 180)	Group 3 (*n* = 180)	Group 4 (*n* = 180)	*P* value
Age (yr)	mean ± SD	41.4 ± 12.6	39.6 ± 13.6	41.4 ± 12.3	41.4 ± 13.5	0.470
Gender	Male	105 (58)	118 (66)	102 (57)	107 (58)	0.337
	Female	75 (42)	62 (34)	78 (43)	73 (42)	

Patients in Group 1 and Group 3 received rabeprazole (10 mg), amoxicillin (1000 mg) and furazolidone (100 mg) twice daily for 7 and 10 d, respectively and patients in Group 2 and Group 4 received rabeprazole (10 mg), bismuth (220 mg), amoxicillin (1000 mg) and furazolidone (100 mg) twice daily for 7 and 10 d, respectively.

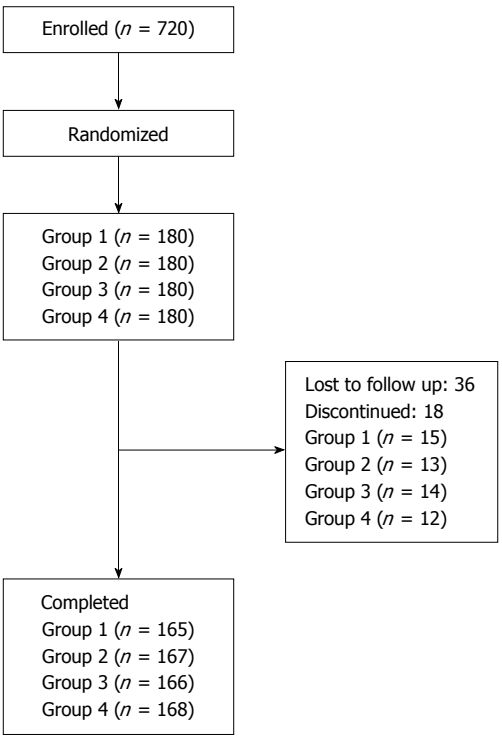

Figure 1 Study flow chart. Patients in Group 1 and Group 3 received rabeprazole (10 mg), amoxicillin (1000 mg) and furazolidone (100 mg) twice daily for 7 and 10 d, respectively. Patients in Group 2 and Group 4 received rabeprazole (10 mg), bismuth (220 mg), amoxicillin (1000 mg) and furazolidone (100 mg) twice daily for 7 and 10 d, respectively.

completed the scheme, with 165, 167, 166 and 168 patients in Groups 1, 2, 3, and 4, respectively (Figure 1).

H. pylori eradication rate

There were significant differences in the *H. pylori* eradication rate (ITT: $\chi^2 = 8.725$, $P = 0.033$; PP: $\chi^2 = 10.094$, $P = 0.018$) among the four groups. Further comparisons revealed that the *H. pylori* eradication rate in Group 4 was significantly higher than that of Group 1 ($P < 0.05$) (Table 2) according to the ITT and PP analyses.

Adverse events

The major adverse events included dizziness, vomiting, diarrhea, nausea, skin rash, itchy skin, and malaise. The number of adverse events was 15/180 (8.3%), 16/180 (8.9%), 15/180 (8.3%) and 17/180 (9.4%) in Groups 1, 2, 3 and 4, respectively (Table 3). All 18 patients with vomiting, skin rash and itchy skin withdrew from the study treatment, including four patients in Group 1 (three patients with vomiting and one patient with skin rash and itchy skin), five patients in Group 2 (four patients with vomiting and one patient with skin rash and itchy skin), four patients in Group 3 (all vomiting), and five patients in Group 4 (three patients with vomiting and two patients with skin rash and itchy skin). The symptoms were relieved without special treatment in all of the patients (Table 3).

DISCUSSION

This study demonstrated that patients treated with a combination of rabeprazole, bismuth, amoxicillin and furazolidone for 10 d (Group 4) had the highest eradication rate of *H. pylori* infection. In addition, the *H. pylori* eradication rates of the four groups were all higher than those of the reports mentioned previously[12,13]. The eradication of *H. pylori* infection was not influenced by the age and/or gender of the patients. In this study, the main adverse events were dizziness, vomiting and diarrhea (Table 3), and the incidence of these side effects was < 10%. These symptoms disappeared after discontinuation of the treatment drugs and did not require any further management. All of the treatment regimens were safe, as serious life threatening side effects were not reported and the incidence was similar in different groups. The majority of patients complied with treatment regimens in different groups, and the patient drop-out rate was not due to the different treatment regimens.

Although *H. pylori* is sensitive to many antibiotics, the treatment effects on *H. pylori* are not satisfactory compared with other bacterial infectious diseases[1]. Graham *et al*[1] in 2007 proposed that the *H. pylori* eradication rate should be greater than 95%, and less than 80% was not acceptable. An increasing resistance rate to clarithromycin and metronidazole has resulted in gradually decreasing *H. pylori* eradication rates of standard "triple therapy" (PPI, clarithromycin, amoxicillin or metronidazole). In 2010, Graham *et al*[14] found that only 18% of the standard "triple therapies" had an eradication rate of more than 85%, while 60% of the ITT analyses showed that the eradication rate could not reach 80%.

A multi-center study in Europe[12] showed that the *H. pylori* eradication rates of a standard triple therapy (omeprazole, amoxicillin, clarithromycin) for 7 d were 70% and 55% according to PP and ITT analyses, respectively. Another multi-center study conducted in Spain by

Table 2 Eradication of Helicobacter pylori infection in the different treatment groups in the intention-to-treat and per protocol analyses *n* (%)

Efficacy	Group 1 (*n* = 180)	Group 2 (*n* = 180)	Group 3 (*n* = 180)	Group 4 (*n* = 180)	χ^2	*P* value
Eradication success	134 (74.4)	149 (82.8)	142 (78.9)	155 (86.1)		
Eradication failure	31 (17.2)	18 (10.0)	24 (13.3)	13 (7.2)		
Cases dropped	15 (8.3)	13 (7.2)	14 (7.8)	12 (6.7)		
Eradication rate						
ITT (95%CI)	74.4% (68.0-80.8)	82.8% (77.3-88.3)	78.9% (72.9-84.9)	86.1%[a] (81.0-91.2)	8.725	0.033
PP (95%CI)	81.2% (75.2-87.2)	89.2% (84.5-93.9)	85.5% (80.1-90.9)	92.3%[a] (88.3-96.3)	10.094	0.018

[a]$P < 0.05$ *vs* Group 1. Patients in Group 1 and Group 3 received rabeprazole (10 mg), amoxicillin (1000 mg) and furazolidone (100 mg) twice daily for 7 and 10 d, respectively and patients in Group 2 and Group 4 received rabeprazole (10 mg), bismuth (220 mg), amoxicillin (1000 mg) and furazolidone (100 mg) twice daily for 7 and 10 d, respectively. ITT: Intention-to-treat; PP: Per protocol.

Table 3 Adverse events in the patients of the four groups *n* (%)

Adverse events	Group 1 (*n* = 180)	Group 2 (*n* = 180)	Group 3 (*n* = 180)	Group 4 (*n* = 180)
Dizziness	5 (2.8)	4 (2.2)	6 (3.3)	7 (3.9)
Vomiting	3 (1.7)	4 (2.2)	4 (2.2)	3 (1.7)
Diarrhea	1 (0.6)	4 (2.2)	2 (1.1)	3 (1.7)
Nausea	3 (1.7)	0 (0)	0 (0)	1 (0.6)
Skin rash and itchy skin	1 (0.6)	1 (0.6)	0 (0)	2 (1.1)
Asthenia	2 (1.1)	3 (1.7)	3 (1.7)	1 (0.6)
Total	15 (8.3)	16 (8.9)	15 (8.3)	17 (9.4)

Patients in Group 1 and Group 3 received rabeprazole (10 mg), amoxicillin (1000 mg) and furazolidone (100 mg) twice daily for 7 and 10 d, respectively and patients in Group 2 and Group 4 received rabeprazole (10 mg), bismuth (220 mg), amoxicillin (1000 mg) and furazolidone (100 mg) twice daily for 7 and 10 d, respectively. Treatment was discontinued for 18 patients who experienced vomiting, skin rash and itchy skin.

Gisbert *et al*[13] showed that the *H. pylori* eradication rates of standard triple therapy (20 mg omeprazole *bid*, 500 mg clarithromycin *bid*, and 500 mg metronidazole *bid*) for 7 d were 55% and 54% according to PP and ITT analyses, respectively. Furthermore, a multi-center clinical study in China showed that the *H. pylori* eradication rates of standard triple therapy (20 mg lansoprazole *bid*, 500 mg clarithromycin *bid*, and 1000 mg amoxicillin *bid*) for 7 d were 78.2% and 74.5% according to PP and ITT analyses, respectively[15]. Another study at the Shanghai Renji Hospital[16] in China showed that the *H. pylori* eradication rates of standard triple therapy (40 mg pantoprazole *bid*, 1 g amoxicillin *bid*, and 500 mg clarithromycin *bid*) for 7 d were 65.1% and 63.5% according to PP and ITT analyses, respectively.

In recent years, numerous studies have shown that the rate of resistance of *H. pylori* to furazolidone is low[17,18]. Moreover, our previous studies over the last 15 years in the Jiangxi province have shown that *H. pylori* resistance rates to metronidazole and clarithromycin gradually increased to 72.7% and 14.9%, respectively, and the resistance rate to levofloxacin was 14.1%. However, the resistance rate to furazolidone was 0%[7]. The present study demonstrated that furazolidone-based 7- and 10-d triple therapies, irrespective of the course of treatment, achieved *H. pylori* eradication rates above the accepted threshold of 80%[2] (81.2% and 85.5%, respectively) by PP analysis. More importantly, the eradication rates of the 7- and 10-d quadruple therapies were also higher than 80% (89.2% and 92.3%, respectively) by PP analysis, which are similar to or even higher than recent reports[12,13,15,16]. Furthermore, the *H. pylori* eradication rate in furazolidone-based quadruple therapy was higher than that of the triple therapy, and the eradication rates of the 10-d therapy were higher than those of the 7-d therapy, suggesting that either the addition of bismuth or an extended course of treatment could improve the *H. pylori* eradication rate.

A prospective study in Iran by Agah *et al*[19] reported that the *H. pylori* eradication rates of metronidazole-based quadruple therapy (500 mg metronidazole *bid*, 1 g amoxicillin *bid*, 20 mg omeprazole *bid*, and 240 mg bismuth *bid*) and azithromycin-based quadruple therapy [500 mg azithromycin once daily (*qd*) for 1 wk, 1 g amoxicillin *bid*, 20 mg omeprazole *bid*, and 240 mg bismuth *bid*] for two weeks were 68% and 69%, respectively, according to PP and ITT analyses. In the present study, the length of the furazolidone-based quadruple therapy was shorter than that of the study by Agah *et al*[19], but the *H. pylori* eradication rate was higher in our study. In the setting of second-line therapy, Kuo *et al*[20] treated 150 patients in Taiwan with levofloxacin-containing quadruple therapy [40 mg of esomeprazole *bid*, 300 mg of bismuth subcitrate four times daily (*qid*), 500 mg tetracycline *qid*, and 500 mg levofloxacin *qd*) or high-dose metronidazole-based quadruple therapy (40 mg esomeprazole *bid*, 300 mg bismuth subcitrate *qid*, 500 mg tetracycline *qid*, and 500 mg metronidazole *qid*) for 10 d. The ITT analysis indicated that the eradication rate was 78.9% and 79.7%, respectively, and the PP analysis indicated that the eradication rate was 87.0% and 90.8%, respectively. When used with furazolidone, Abbas *et al*[21] treated 52 patients in Pakistan who failed to respond to clarithromycin-based triple therapy with a combinational regimen comprised of furazolidone (200 mg), co-amoxiclav (1 g), colloidal bismuth subcitrate (240 mg) and esomeprazole (40 mg) *bid* for 14 d. To document eradication of *H. pylori*, the urea breath test was repeated 4 wk after the completion of treatment. The ITT analysis indicated that the eradication rate was 81% (42/52), and the PP analysis indicated that the eradication rate was 82.4% (42/51)[15]. Another study from

Iran also showed good outcomes for *H. pylori* eradication with bismuth-based therapy using furazolidone, revealed eradication rate of 80.6% and 82.9%, according to ITT and PP analyses[22].

The therapeutic dose of rabeprazole used in this study was 10 mg twice a day. Rabeprazole has proven efficacy in healing, symptom relief and prevention of relapse of peptic ulcers and GORD. As monotherapy for peptic ulcer healing and symptom relief, 4- to 8-wk studies have shown that the efficacy of rabeprazole (10 to 40 mg *qd*) is similar to omeprazole[23]. A rabeprazole dose of 10 mg *bid* is recommended by the Fourth Chinese National Consensus Report[3], and the recommended dose of rabeprazole by the Maastricht IV Consensus[2] is 20 mg *bid*. In this study, the rabeprazole dose was increased to 20 mg *bid* in those who failed eradication treatment.

The adverse events of furazolidone are associated with its dose and duration of use. Various studies have indicated that the incidence of adverse events at a higher dose of furazolidone (200 mg *bid*) is greater than that at a lower dose (100 mg *bid* or 50 mg *bid*). In this study, the dose of furazolidone was 100 mg *bid* (lower dose), and the course of treatment was 10 d or less. Adverse events were observed in less than 10% of patients in each of the groups, and treatment had to be discontinued in 2.5% of the patients.

Chinese national multicenter randomized controlled trials have indicated that sequential therapy has no significant advantage when compared with standard triple therapy[24]. Accompanying therapy may increase antibiotic adverse reactions, as well as reducing options of antibiotics after eradication treatment failure. Furazolidone is seldom used in the United States and European countries, but it is used widely in several countries, including Iran, Malaysia, Pakistan, Brazil, Mexico and China. The infection rate of *H. pylori* in the Asian-Pacific region is very high. Furazolidone-based therapy is safe and our results have shown that the side effects of both 7-d and 10-d treatments were less than 10%. In a future study, we will expand the sample size to assess furazolidone-based therapy around other areas of China.

In conclusion, both 7- and 10-d quadruple furazolidone-based therapies achieve satisfactory *H. pylori* eradication rates. Therefore, these regimens are recommended as an alternative treatment for *H. pylori* eradication.

COMMENTS

Background

The eradication rate of *Helicobacter pylori* (*H. pylori*) should be higher than 90%. However, in recent years, the widespread use of antibiotics has led to the development of drug resistance to antibiotics used in the treatment of *H. pylori* infection. The eradication rate of *H. pylori* infection has fallen to less than 80% with standard triple therapy (proton pump inhibitor, clarithromycin and amoxicillin as a first-line regimen).

Research frontiers

A number of interesting articles have been published over the last year assessing many issues around *H. pylori* eradication therapy, including triple therapy, nonbismuth quadruple therapies, and bismuth-based therapy, often with conflicting outcomes.

Innovations and breakthroughs

This study demonstrated that both 7- and 10-d quadruple furazolidone-based therapies achieve satisfactory *H. pylori* eradication rates, and all of the treatment regimens are safe. Therefore, these regimens are recommended as an alternative treatment for *H. pylori* eradication.

Applications

Furazolidone is seldom used in the United States and European countries, but it is used widely in countries in the Asian-Pacific region, including Iran, Malaysia, Pakistan, Brazil, Mexico and China, in which the infection rate of *H. pylori* is very high. Furazolidone-based therapy is efficient, safe and cost-effectiveness, and is suitable as an alternative treatment for *H. pylori* eradication, especially in developing countries.

Peer review

The manuscripts studied furazolidone-based triple and quadruple treatment in *H. pylori* infected patients with duodenal ulcer. The study is well prepared and the results are specific, especially on the basis of cost-effectiveness.

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P- Reviewer: Buzas GM, Lai YC, Pimanov SI, Yokota S
S- Editor: Gou SX **L- Editor**: O'Neill M **E- Editor**: Ma S

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DOI: 10.3748/wjg.v20.i32.11422

World J Gastroenterol 2014 August 28; 20(32): 11422-11428
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

RANDOMIZED CLINICAL TRIAL

Efficacy of tandospirone in patients with irritable bowel syndrome-diarrhea and anxiety

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Received: December 22, 2013 Revised: April 3, 2014
Accepted: May 28, 2014
Published online: August 28, 2014

Abstract

AIM: To investigate the efficacy of tandospirone in patients with irritable bowel syndrome-diarrhea (IBS-D) and anxiety in a prospective, randomized, controlled study.

METHODS: Two hundred patients with IBS-D and moderate anxiety were randomized to receive pinaverium and tandospirone (arm A) or pinaverium and placebo (arm B). Tandospirone or placebo was given thrice daily at a fixed dose of 10 mg and pinaverium was given thrice daily at a fixed dose of 50 mg. The duration of treatment was 8 wk. Patients were assessed for abdominal pain and diarrhea. Anxiety was evaluated using the Hamilton Rating Scale for Anxiety (HAM-A). The primary study endpoints were response rates for abdominal pain and diarrhea. The secondary study endpoints were response rates for anxiety. Adverse events were also evaluated.

RESULTS: One hundred and seventy of 200 patients (82 patients in arm A and 88 patients in arm B) completed the study. Demographic and baseline characteristics of the 200 participants were comparable in the two arms. At week 8, the overall response rate for abdominal pain and diarrhea was 52.0% for arm A and 37.0% for arm B ($P < 0.05$). The HAM-A score showed that the response rate was 61.0% for arm A and 21.0% for arm B ($P < 0.01$). The treatments were well tolerated and no significant adverse events were reported.

CONCLUSION: Tandospirone is effective and can be combined with pinaverium in IBS-D patients with anxiety.

Key words: Irritable bowel syndrome; Anxiety; Tandospirone; Efficacy; Safety

Core tip: Irritable bowel syndrome (IBS) is associated with psychological stress, anxiety and depression, which may contribute to perpetuating the condition. IBS-diarrhea (IBS-D), an isotype of IBS, is often accompanied by anxiety, and conventional therapy is unfavorable. IBS-D may respond positively to anti-anxiety/-depression therapies. However, existing medications are not sufficiently effective for patients with IBS-D. To our knowledge, few randomized, controlled and multicenter studies have focused on the efficacy of anti-anxiety agents in IBS-D patients. This is a prospective, randomized, controlled study to evaluate the efficacy of tandospirone in patients with combined IBS-D and anxiety.

Lan L, Chen YL, Zhang H, Jia BL, Chu YJ, Wang J, Tang SX, Xia GD. Efficacy of tandospirone in patients with irritable bowel

syndrome-diarrhea and anxiety. *World J Gastroenterol* 2014; 20(32): 11422-11428 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11422.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11422

INTRODUCTION

Irritable bowel syndrome (IBS) is one of the most common gastrointestinal disorders (GIDs) with major symptoms such as abdominal discomfort and pain accompanied by constipation or diarrhea. The syndrome has complex mechanisms and is usually refractory to treatment[1]. IBS is associated with psychological stress, anxiety and depression, which may contribute to perpetuating the condition[2-4], and may respond positively to anti-anxiety/-depression therapies[5-7]. However, existing medications are not sufficiently effective for patients with IBS.

IBS-diarrhea (IBS-D) is the most frequent subtype of IBS[8]. IBS-D patients usually suffer from anxiety[9]. Anti-anxiety agents should be effective in relieving anxiety and symptoms of IBS-D. However, there have been few randomized and controlled trials of anti-anxiety agents in IBS-D patients.

Tandospirone citrate is a partial agonist of the 5-hydroxytryptamine 1A (5-HT1A) receptor and has also demonstrated neuropharmacological properties that may contribute to its efficacy in the treatment of anxiety[10,11]. However, there have been no studies on the efficacy and safety of tandospirone in IBS-D patients with anxiety. We conducted a prospective, multicenter, single-blind, randomized, controlled study to evaluate the efficacy (whether abdominal pain, diarrhea and anxiety could be improved) of tandospirone in patients with IBS-D and anxiety.

MATERIALS AND METHODS

Participants

This study enrolled patients from three tertiary care centers in China: The People's Hospital and the First Affiliated Hospital of Zhengzhou University, Henan, China and the First Affiliated Hospital of Luzhou Medical College, Sichuan, China. This study was conducted from March 2011 to May 2013. IBS-D was diagnosed according to the Rome Ⅲ criteria (mainly including abdominal pain, diarrhea and without any organic alteration)[12], and anxiety was diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders-Fourth edition (DSM-Ⅳ) criteria[13].

Patients were eligible for enrollment if they (1) were aged between 18 and 65 years; (2) had a Hamilton Rating Scale for Anxiety (HAM-A) score between 14 and 24 (moderate anxiety)[14]; (3) had negative routine fecal and occult blood test within 3 mo prior to the study; (4) had no organic diseases by enteroscopy within three months prior to study, or no hepatobiliary and pancreatic diseases by laboratory studies and ultrasonographic evaluations; and (5) received no agents that influence motility of the gastrointestinal tract and digestion and/or anti-anxiety/depressive drugs within 4 wk prior to study entry. Major exclusion criteria were (1) allergy to tandospirone and/or pinaverium bromide; (2) the presence of functional dyspepsia (FD); (3) breast-feeding or pregnancy or be going to be pregnant in the period of the study; (4) clinically significant diseases or any psychiatric disorder other than anxiety; (5) previous abdominal surgery; and (6) daily alcohol consumption > 40 g and/or history of drug abuse. Patients were also excluded if they used other psychotropic medications in the previous week (14 d for monoamine oxidase inhibitors and 28 d for fluoxetine), or required ongoing use of psychotropic medications.

All participating institutions had implemented good clinical practice and were eligible for conduction of clinical trials. The study was compliant with the Declaration of Helsinki, and approved by local ethical committees and institutional review boards at the participating institutions. All study participants or their legal surrogates provided written informed consent.

Study intervention

Eligible subjects were blinded to this study and randomized at an allocation ratio of 1:1 to receive pinaverium (a calcium channel blocker for helping to restore the normal contraction process of the bowel) and tandospirone (arm A) or pinaverium and placebo (arm B). Tandospirone or placebo was given thrice daily at a fixed dose of 10 mg and pinaverium was given thrice daily at a fixed dose of 50 mg. The duration of treatment was 8 wk. Patients who missed more than five consecutive days of treatment in 8 wk of the study (non-compliant) were withdrawn from the trial.

Patient evaluation

Patients were assessed for eligibility at a screening visit, with eligible patients returning for a baseline assessment approximately one week, and then evaluated at week 8 for a total of four visits. The primary study endpoints were response rates for abdominal pain and diarrhea. The secondary study endpoints were the response rates for anxiety.

Abdominal pain was assessed using a 10-point abdominal pain numeric rating scale (NRS) from 0 (none) to 10 (worst possible pain), and mild (NRS score, 1-3), moderate (NRS score, 4-6) and severe pain (NRS score, 7-10) were then assigned a score of 1, 2 or 3, respectively. In addition, the frequency of abdominal pain was assigned a score of 0, 1, 2 and 3, respectively, if the pain occurred 0, 1-2, 3-4 and ⩾ 5 times per week. The abdominal pain score represented the sum of the severity and frequency of pain in a patient. Diarrhea was assessed by stool consistency, frequency and urgency. Normal/hard feces, roughly normal feces, soft feces, loose feces and watery feces were assigned a score of 0, 1, 2, 3 or 4, respectively. Defecations were assigned a score of 0, 1,

2, 3 or 4 if they occurred ≤ 1-2, 3-4, 5-6 and ≥ 7 times daily. The absence or presence of urgency was assigned a score of 0 or 1. The diarrhea score represented the combination of scores for stool consistency, frequency and urgency of a patient. Furthermore, anxiety was evaluated using the HAM-A scale.

For abdominal pain and diarrhea, clinical response was evaluated based on the treatment associated-reduction rate (TARR), which was defined as the post-treatment scores minus the pretreatment scores and then divided by the pretreatment scores and multiplied by 100%. Complete response (CR) had a TARR ≥ 75%, partial response (PR) had a TARR ≥ 50% but <75%, slight response (SR) had a TARR ≥ 25% but < 50% and non-response (NR) had a TARR < 25%. The response rate = $CR_{\text{Abdominal pain or diarrhea}}$ + $PR_{\text{Abdominal pain or diarrhea}}$. The overall response rate = CR (abdominal pain plus diarrhea) + PR (abdominal pain plus diarrhea). Clinical response in anxiety was evaluated using the same methods as those for abdominal pain and diarrhea.

Evaluation of adverse events

Adverse events were monitored at baseline and week 2 and 8 using the Treatment Emergent Symptom Scale (TESS) (NIMH, 1973). Safety assessments were based mainly on the occurrence, frequency, and severity of adverse events and were also based on laboratory parameters including hematology, hepatorenal function, electrolytes, urinalysis, fecal tests and electrocardiography, and treatment-emergent adverse events were recorded. For all adverse events, where necessary, patients were withdrawn from the study.

Statistical analysis

Sample size calculation was based on the assumption of a 40% response in the arm A *vs* 20% in arm B using the *Z* statistic to compare dichotomous variables with $\alpha = 0.05$ (two-tailed) and $\beta = 0.20$. The estimated sample size was 81 patients per arm.

Randomization procedures were performed using a computer code generated by a study statistician who did not have contact with the study subjects. Trial name and all involved parameters were set. Participating centers were pre-added in the system. Common users of the system at each center were granted corresponding permission and user-names in advance. They accessed the system, input patient demographic data, and selected items according to the inclusion/exclusion criteria. The system then automatically decided whether a patient was eligible to participate in the study.

The statistical analysis was carried out using SPSS14.0 software. The statistical analyses were pre-specified and performed on an intention-to-treat basis with the inclusion of all patients who underwent randomization. Both full and per-protocol analyses were used. The full analysis sets included all patients who were randomized to treatment and had a baseline assessment and at least one post-baseline assessment. The per-protocol sets included all evaluable patients who completed at least three weeks of active treatment and were not excluded as protocol violators. Unless otherwise specified, all efficacy results reported herein are based on the full analysis, whereas, for patients who withdrew or were lost to follow-up, we used the last observation carried forward approach. Descriptive statistics were used to summarize some safety measures. The χ^2 test was used in the statistical analysis and $P < 0.05$ was considered statistically significant. The homogeneity of the HAM-A score obtained from different investigators was analyzed using Kendall's *W* test, with $W > 90\%$ considered as having homogeneity.

RESULTS

Demographic and baseline characteristics of the participants

The study flowchart is shown in Figure 1. Among 274 subjects screened, 200 were eligible for the study. One hundred patients were assigned to receive tandospirone and pinaverium, and 100 to receive tandospirone. Thirty patients (18 in arm A and 12 in arm B) withdrew due to adverse events, lack of efficacy, protocol violation, lost to follow-up or withdrawal consent. In total, 82 patients in arm A and 88 patients in arm B completed the study. Demographic and baseline characteristics of the 170 participants are shown in Table 1. The median age of the study subjects was 45.6 (range: 19-65) years and there were slightly more male patients (54.1%, 92/170) than female patients (45.9%, 78/170). Patients in the two arms were well balanced in demographic characteristics. The mean baseline HAM-A score was 21.8 ± 5.2 for arm A and 20.9 ± 4.7 for arm B with no apparent difference between the two arms ($P = 0.21$). The mean baseline abdominal pain and diarrhea score was comparable between arm A (8.3 ± 2.0) and arm B (8.0 ± 2.1) ($P = 0.18$).

Primary study endpoints

Forty-three patients in arm A and twenty-nine patients in arm B had a 50% or greater reduction in the abdominal pain score at week 8. The response rate was 43.0% for arm A and 29.0% for arm B in the intention-to-treat population ($P < 0.05$), and was 52.4% for arm A and 33.0% for arm B in the per-protocol sets ($P < 0.05$) (Table 2).

Fifty patients in arm A and 34 patients in arm B had a 50% or greater reduction in the diarrhea score at week 8. The response rate was 50.0% for arm A and 34.0% for arm B in the intention-to-treat population ($P < 0.05$), and was 61.0% for arm A and 38.6% for arm B per-protocol sets ($P < 0.01$) (Table 2).

The overall response rate in patients with abdominal pain and diarrhea was 52.0% for arm A and 37.0% for arm B in the intention-to-treat population ($P < 0.05$), and was 63.4% for arm A and 42.0% for arm B in the per-protocol sets ($P < 0.01$) (Table 2).

Secondary study endpoints

The Kendall coefficient was > 90%, indicating the ho-

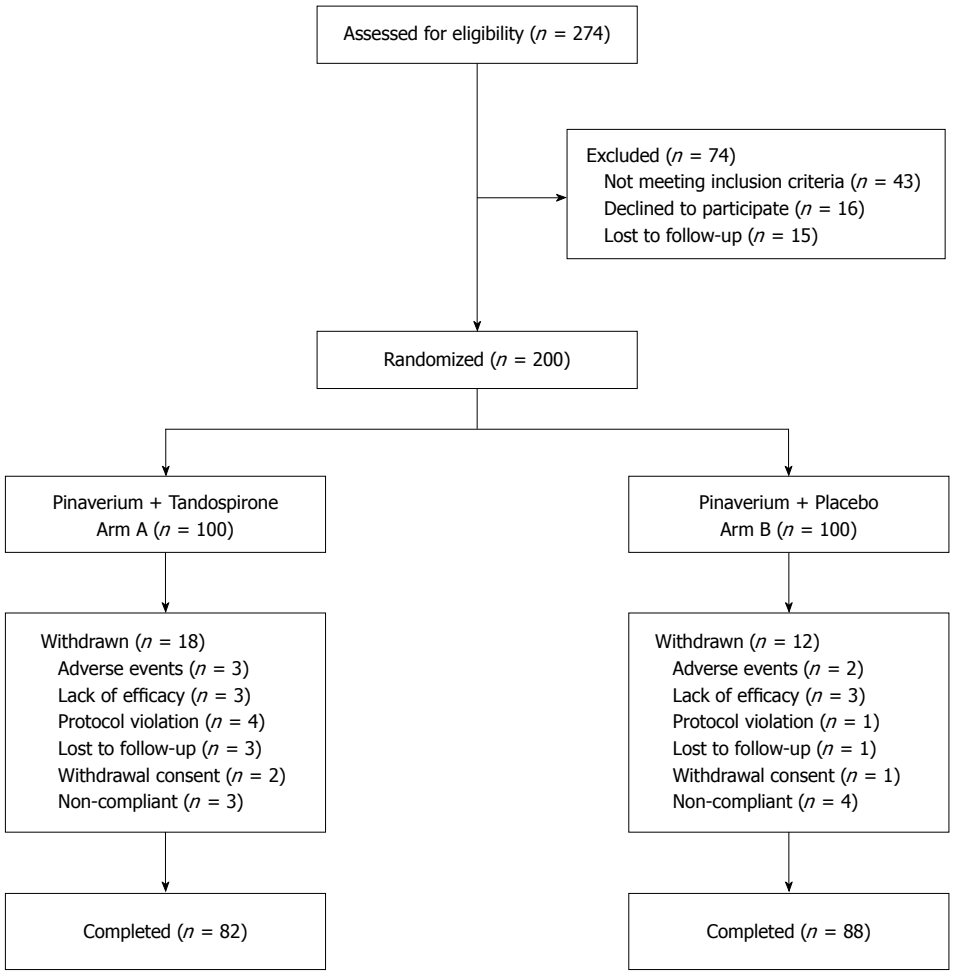

Figure 1 CONSORT diagram showing the flow of subjects in each stage of the trial.

Table 1 Demographic and baseline characteristics of the study participants with irritable bowel syndrome-diarrhea *n* (%)

Characteristic	Arm A (*n* = 100)	Arm B (*n* = 100)
Age (yr)		
mean ± SD	46.6 ± 12.9	44.7 ± 12.8
Range	20-65	19-64
Female gender	35 (42.7)	43 (48.9)
Anxiety		
mean ± SD	21.8 ± 5.2	20.9 ± 4.7
Range	14-24	14-24
Abdominal pain and diarrhea score		
mean ± SD	8.3 ± 2.0	8.0 ± 2.1
Range	4-12	4-14

mogeneity of the HAM-A score among investigators. Sixty-one patients in arm A and 21 patients in arm B had a 50% or greater reduction in the HAM-A score at week 8. The response rate was 61.0% for arm A and 21.0% for arm B in the intention-to-treat population ($P < 0.01$), and was 74.4% for arm A and 23.9% for arm B in the per-protocol sets ($P < 0.01$) (Table 3).

Adverse events

The treatments were well tolerated and no significant adverse events were reported. Treatment-emergent adverse events included somnolence (four in arm A), vertigo (two in arm A), vomiting (one in arm A and one in arm B) and aggravated abdominal pain (one subject in arm B), which resulted in discontinuation (vertigo, vomiting and aggravated abdominal pain) or was resolved two weeks later (somnolence).

DISCUSSION

IBS is a functional gastrointestinal disorder (FGID) characterized by symptoms including abdominal pain, distention and abnormal defecation habit and feces appearance[8]. Despite absence of organic disease, IBS may have a notable adverse effect on the quality of life of patients and lead to the exhaustion of medical resources[15]. The etiology of IBS is unknown. However, it has been demonstrated that IBS is the GID which was most strongly associated with mental health conditions[16]. Mental stress and psychological distress are correlated with development of IBS[17]. Psychological and social factors can interfere with the communication between the central and enteric nervous systems, and there is proof that they are involved in the onset of IBS and influence the response to treatment and outcome[18]. Anxiety or depres-

Table 2 Response rates in the subjects with irritable bowel syndrome-diarrhea for abdominal pain and diarrhea *n* (%)

	Arm	*n*	CR	PR	SR	NR	Response rate
Abdominal pain							
Per protocol	A	82	22 (26.8)	21 (25.6)	27 (32.9)	12 (14.6)	43 (52.4)[a]
	B	88	14 (15.9)	15 (17.0)	28 (31.8)	31 (35.2)	29 (33.0)
Intention-to-treat	A	100	22 (22)	21 (21)	27 (27)	30 (30)	43 (43)[a]
	B	100	14 (14)	15 (15)	28 (28)	43 (43)	29 (29)
Diarrhea							
Per protocol	A	82	25 (30.5)	25 (30.5)	23 (28)	9 (11)	50 (61.0)[b]
	B	88	13 (14.8)	21 (23.9)	30 (34.1)	24 (27.3)	34 (38.6)
Intention-to-treat	A	100	25 (25)	25 (25)	23 (23)	27 (27)	50 (50)[a]
	B	100	13 (13)	21 (21)	30 (30)	36 (36)	34 (34)
Abdominal pain plus diarrhea							
Per protocol	A	82	24 (29.3)	28 (34.1)	21 (25.6)	9 (11)	52 (63.4)[b]
	B	88	11 (12.5)	26 (29.5)	15 (17.0)	36 (40.9)	37 (42.0)
Intention-to-treat	A	100	24 (24)	28 (28)	21 (21)	27 (27)	52 (52)[a]
	B	100	11 (11)	26 (26)	15 (15)	48 (48)	37 (37)

[a]$P < 0.05$, [b]$P < 0.01$ *vs* arm B. CR: Complete response; NR: Non-response; PR: Partial response; SR: Slight response.

Table 3 Response rates in the subjects with irritable bowel syndrome-diarrhea for anxiety *n* (%)

Anxiety	Arm	*n*	CR	PR	SR	NR	Response rate
Per protocol	A	82	16 (19.5)	45 (54.9)	17 (20.7)	4 (4.9)	61 (74.4)[b]
	B	88	4 (4.5)	17 (19.3)	25 (28.4)	42 (47.7)	21 (23.9)
Intention-to-treat	A	100	16 (16)	45 (45)	17 (17)	22 (22)	61 (61)[b]
	B	100	4 (4)	17 (17)	25 (25)	54 (54)	21 (21)

[b]$P < 0.01$, arm A *vs* arm B. CR: Complete response; NR: Non-response; PR: Partial response; SR: Slight response.

sion may influence autonomic nervous system balance in women with IBS[19]. Gastrointestinal (GI)-specific anxiety seems to be an important factor in GI symptom severity and quality of life in patients with IBS[20]. No effective therapy is currently available for IBS-D, and commonly used medications including pinaverium and trimebutine are unfavorable in refractory IBS and are associated with frequent recurrences. Patients with refractory IBS usually experience negative mood and sleep disturbance[21], indicating the importance of psychological intervention and anti-anxiety/-depressive therapy in the treatment of IBS[22-25].

As a third-generation anti-anxiety agent, tandospirone is a novel partial agonist of 5-HT$_{1A}$ receptor and modulates 5-HT projected from the raphe nuclei to the hippocampus by selectively activating 5-HT$_{1A}$ receptor in a postsynaptic manner, thus exerting its anti-anxiety activity[26]. In addition, tandospirone has an anti-depressive effect by down-regulating presynaptic 5-HT$_{1A}$ receptor density[27]. Therefore, tandospirone has dual anti-anxiety and anti-depressive effects, particularly in anxiety and takes effect at 1 to 2 wk after administration. 5-HT$_{1A}$ receptor is located at the cholinergic nerve terminal and the presynaptic component of the neuromuscular junction, and may lead to relaxation of smooth muscle when it is activated[28,29]. Therefore, tandospirone can not only act on psychological symptoms including anxiety and depression, but also improve autonomic nerve disorder-related physical symptoms, such as abnormalities of appetite, sexual behavior, body temperature and blood pressure[30]. Therefore it can be used to treat eating and GI disorders[31,32]. As reported previously[28,32,33], tandospirone can be used in patients with combined functional dyspepsia and emotional disorders, particularly those with treatment-refractory FD.

This study demonstrated that the combination of tandospirone and pinaverium was associated with a significantly increased overall response rate in patients with abdominal pain and diarrhea. Furthermore, we found that the combination of tandospirone and pinaverium was associated with a markedly higher response rate. These findings demonstrate that tandospirone is effective in patients with IBS-D with anxiety. Fukudo *et al*[34] showed that IBS is related to brain-gut interactions, emotional dysregulation, and illness behaviors. Corticotropin-releasing hormone and 5-HT are candidate substances which regulate exaggerated brain-gut response. Therefore, it is possible that tandospirone as a partial 5-HT$_{1A}$ agonist, can regulate brain-gut response and have an anti-spasmodic effect on the colon by binding 5-HT$_{1A}$ receptor, thereby producing an improvement in abdominal pain and diarrhea.

We also found that the drug was overall well tolerated by IBS-D patients and showed a benign safety profile with no major treatment-emergent adverse events. This compares favorably to selective serotonin reuptake inhibitors (SSRIs), anti-anxiety/-depressive agents used in the clinic, which also exert their effects *via* the 5-HT system. However, as an SSRI blocks the reuptake of

5-HT, thus increasing 5-HT levels in the synaptic cleft, this may lead to agitation and increase the frequency of adverse events[35]. In addition, tandospirone is associated with less somnolence and dependency than other anti-anxiety agents due to the absence of non-anti-anxiety effects associated with benzodiazepines, such as muscular relaxation, anti-convulsion and sedation[28], and can be withdrawn when the symptoms are resolved[22].

Clinically, IBS is primarily treated with drugs acting on GI motility, spasmolysis and analgesia, and psychological disorders are suspected only if the above-mentioned treatments are ineffective. However, in IBS patients, as an interaction between psychological disorders and physical symptoms may exacerbate the condition, delaying anti-anxiety/-depressive intervention until poor efficacy of conventional treatments is confirmed may extend the distress of these patients[22]. In our opinion, anti-anxiety/-depressive treatment should be administered at an early period for IBS patients who present with anxiety and depression in order to avoid chronic stress[36].

Our prospective randomized controlled multicenter trial has demonstrated that tandospirone is effective and safe in IBS-D patients with anxiety. Prompt anti-anxiety therapy in IBS-D patients with anxiety could lead meaningful improvements in anxiety as well as a significant reduction in abdominal pain and diarrhea. Further larger-scale, long-term clinical trials are warranted to confirm our findings.

ACKNOWLEDGMENTS

We acknowledge all site investigators for their participation in the study: Jing Yu, Chang-He Jia, Bo-Wei Liu, Yuan Yuan. We also thank the Endoscope Center of the People's Hospital of Zhengzhou University, Zhengzhou, China.

COMMENTS

Background

The etiology of irritable bowel syndrome (IBS) is unknown. IBS-diarrhea (IBS-D) is an isotype of IBS. No effective therapy is available for IBS-D, and commonly used medications including pinaverium and trimebutine are unfavorable for refractory IBS and are associated with frequent recurrences. Along with the "biomedical model" changing to the "biopsychosocial medical model", the influence of psychological stress on IBS has been followed with interest.

Research frontiers

Recently, many studies found that IBS is associated with psychological stress, anxiety and depression. IBS-D is often accompanied by anxiety. More researchers are now attempting to determine whether IBS-D may respond to anti-anxiety/-depression therapies.

Innovations and breakthroughs

Few randomized, controlled and multicenter studies have focused on the efficacy of anti-anxiety agents in IBS-D patients. As a third-generation anti-anxiety agent, tandospirone has not been used for the treatment of IBS in studies. The authors conducted a prospective, randomized, controlled study to evaluate the efficacy of tandospirone in patients with IBS-D and anxiety.

Applications

Tandospirone treatment in IBS-D patients with anxiety could lead meaningful improvements in anxiety as well as a significant reduction in abdominal pain and diarrhea. This may provide a novel therapeutic option for IBS-D patients with anxiety.

Peer review

This is a well-written paper. The topic is timely and of general interest. This randomized controlled trial could have contribution to the management of IBS-D.

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P- Reviewer: Henderson P, Kuo SM, Poli-Neto OB
S- Editor: Ma YJ **L- Editor**: Wang TQ **E- Editor**: Ma S

World Journal of Gastroenterology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i32.11429

World J Gastroenterol 2014 August 28; 20(32): 11429-11438
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

META-ANALYSIS

Methylenetetrahydrofolate reductase C677T and A1298C polymorphisms and gastric cancer susceptibility

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Received: October 8, 2013 Revised: March 4, 2014
Accepted: May 12, 2014
Published online: August 28, 2014

Abstract

AIM: To identify the association between methylenetetrahydrofolate reductase (*MTHFR*) polymorphisms and gastric cancer (GC) susceptibility.

METHODS: Systematic searches were performed on the electronic databases PubMed, ISI, Web of knowledge, CNKI and Wanfang, as well as manual searching of the references of the identified articles. A total of 26 papers were included in this meta-analysis. Overall and subgroup analyses were performed. Odds ratio (OR) and 95%CI were used to evaluate the associations between *MTHFR* polymorphisms and GC risk. The I^2 statistics were used to evaluate between-study heterogeneity. Sensitivity analysis was also performed.

RESULTS: Increased risk was found for the MTHFR C677T polymorphism under four genetic models (TT + CT *vs* CC: OR = 1.23, *P* = 0.002; T *vs* C: OR = 1.15, *P* = 0.001; TT *vs* CC: OR = 1.37, *P* = 0.0005; TT *vs* CT + CC: OR = 1.17, *P* = 0.0008). Subgroup analysis by ethnicity suggested that C677T polymorphism conferred a risk of GC in eastern but not in western populations. Stratification by tumor site showed an association between the C677T polymorphism and gastric cardia cancer and non-cardia GC in the worldwide population and in eastern populations. Regardless of comparisons with controls or diffuse-type GC, a positive association was found for the C677T polymorphism and an increased risk of intestinal-type GC in the whole population and in western populations. With regard to the A1298C polymorphism, we found that genotype CC was significantly decreased and conferred protection against GC in eastern populations (CC *vs* AA: OR = 0.44, *P* = 0.03; CC *vs* AC + AA: OR = 0.46, *P* = 0.04).

CONCLUSION: MTHFR C677T polymorphism is a risk factor for GC, and the A1298C polymorphism may be a protective factor against GC in eastern populations.

Key words: Methylenetetrahydrofolate reductase; Polymorphism; Gastric cancer; Meta-analysis

Core tip: Many studies have reported associations of methylenetetrahydrofolate reductase (MTHFR) C677T and A1298C polymorphisms with susceptibility to gastric cancer (GC). There are several relevant published meta-analyses about this subject. Nevertheless, these articles failed to analysis *MTHFR* polymorphisms and GC risk *per se* in detail as follows. They failed to investigate the difference between gastric cardia cancer and non-cardia GC, and the distinction between diffuse and intestinal subtypes. Consequently, we performed a meta-analysis to clarify the roles of MTHFR C677T and A1298C polymorphisms in GC susceptibility among the eligible studies.

Xia LZ, Liu Y, Xu XZ, Jiang PC, Ma G, Bu XF, Zhang YJ, Yu F, Xu KS, Li H. Methylenetetrahydrofolate reductase C677T and A1298C polymorphisms and gastric cancer susceptibility. *World J Gastroenterol* 2014; 20(32): 11429-11438 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11429.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11429

INTRODUCTION

The incidence of gastric cancer (GC) has decreased worldwide, but it remains the fourth most common cancer diagnosis in men and the fifth in women[1], and the second leading cause of cancer-related death[2,3]. The etiology of GC is believed to be multi-stage and multifactorial. Although the decrease in the incidence of GC[4] in recent decades can be explained by changing lifestyles, diet habits, and reduced *Helicobacter pylori* infection, the fact that some individuals develop GC while others do not under similar environmental circumstances suggests that genetic predisposition plays an important role in the pathogenesis of GC.

Methylenetetrahydrofolate reductase (MTHFR), whose gene maps to chromosome 1p36.3[5] and encodes a 77-kDa protein[6], plays a key role in folate metabolism by irreversibly catalyzing the reduction of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the predominant circulatory form of folate, which serves as both a cofactor and substrate for the regeneration of methionine. The latter leads to production of *S*-adenosylmethionine (SAM); the universal methyl donor in humans for DNA methylation[7]. Reduced enzyme activity may result in lower levels of SAM and an increased risk of cancer, including GC, as a consequence of gene hypomethylation[8]. Two common single nucleotide polymorphisms (SNPs) of MTHFR have been indicated: C677T (rs1801133), which results in the amino acid product changing from alanine to valine[9]; and A1298C (rs1801131), which results in the amino acid product changing from glutamic acid to alanine[8]. Studies have confirmed that the variant genotypes are associated with a significant reduction of enzyme activity[10,11], suggesting that the polymorphisms of C677T and A1298C may be related to the risk of GC.

Until now, many studies have reported associations of MTHFR C677T and A1298C polymorphisms with susceptibility to GC with controversial results[12-37]. Additionally, there have been several relevant meta-analyses published on this subject[38-44]. Nevertheless, these studies failed to analyze *MTHFR* polymorphisms and GC risk *per se* in detail as follows. They failed to address the difference between gastric cardia cancer (GCC) and non-cardia gastric cancer (NCGC) as well as the distinction between diffuse and intestinal subtypes. Consequently, we performed a meta-analysis to clarify the roles of MTHFR C677T and A1298C polymorphisms in GC susceptibility among the eligible studies.

MATERIALS AND METHODS

Search strategy

Two researchers independently performed a computerized search in four databases - PubMed, ISI Web of Knowledge (Version 4.5), Chinese National Knowledge Infrastructure, and Wanfang (Chinese) - up to May 2013. Moreover, an additional search was carried out for relevant studies on scholar.google.com.hk. The search terms were "methylenetetrahydrofolate reductase" or MTHFR, "gastric or stomach or cardia" and "cancer or carcinoma or neoplasm" in various combinations, with the language limited to English and Chinese. The reference list of each relevant publication was also reviewed to ensure that all appropriate studies were included in the meta-analysis.

Inclusion and exclusion criteria

Studies were included according to the following criteria: (1) case-control or cohort studies determining the distribution of MTHFR C677T and/or A1298C genotypes; (2) cases with GC were diagnosed by histopathological biopsy, and the controls were free of cancer; and (3) the numbers of cases and controls reported for each genotype should be sufficient for calculation. If multiple studies from the same case series were available, the one including the most individuals was used in the analysis. We excluded the studies if they were: (1) meeting abstracts, case reports, reviews, or editorials; (2) not written in English or Chinese; or (3) not in Hardy-Weinberg equilibrium (HWE) with the controls. The final included studies were based on discussion among the researchers.

Data extraction

Two investigators independently extracted data from the published reports using a standardized protocol and a reporting form with the following information: first author's last name, year of publication, country and ethnicity of participants (classified into eastern and western), sample size, detailed genotype information (genotype distribution and allele frequency), anatomical site of tumor (cardia or non-cardia GC) and Lauren classification (intestinal or diffuse subtype).

Statistical analysis

The MTHFR C677T genotypes include TT, CT and CC, and A1298C comprises CC, AC and AA genotypes. The pooled odds ratios (ORs) were calculated for the dominant model [C677T: (TT + CT) *vs* CC; A1298C: (CC + AC) *vs* AA], the allelic model (C677T: T allele *vs* C allele; A1298C: C allele *vs* A allele), the additive model (C677T: TT *vs* CC; A1298C: CC *vs* AA), and the recessive model (C677T: TT *vs* (CT + CC); A1298C: CC *vs* (AC + AA), respectively. Given that the potential causes of heterogeneity among studies were ethnicity, tumor site and classification, subgroup analyses were conducted according to different ethnic groups (Eastern/Western), tumor site (cardia/non-cardia), and Lauren classification (intestinal/diffuse).

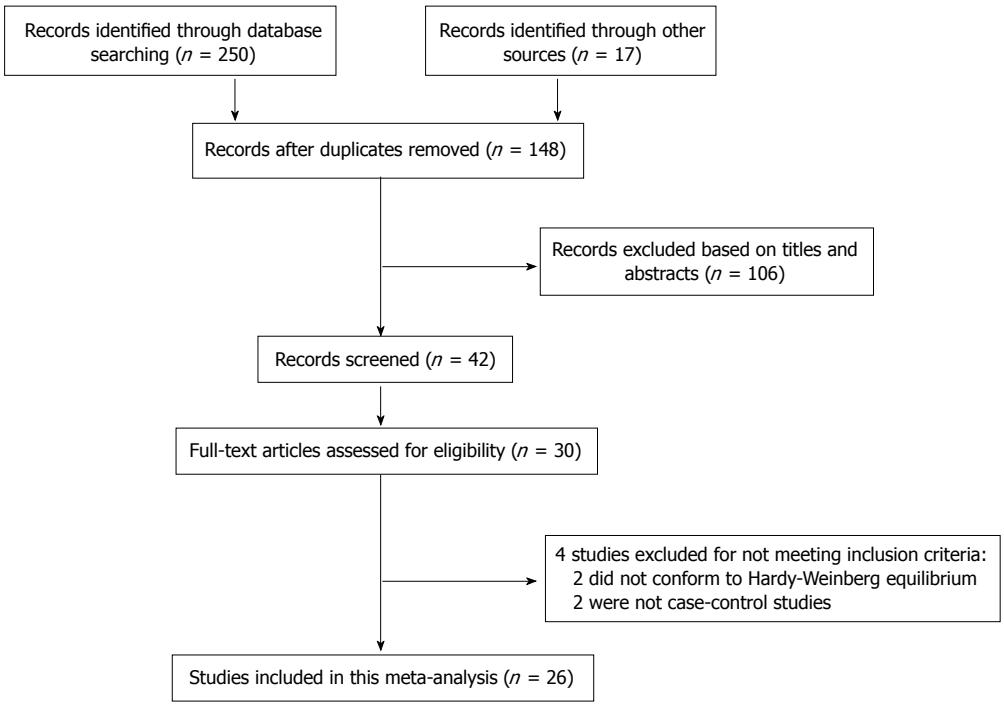

Figure 1 Flowchart of the literature selection process.

RevMan software (Review Manager, Version 5.1; Cochrane Collaboration, 2011) was used for this meta-analysis. The between-study heterogeneity (*i.e.*, the variation in findings not compatible with chance alone) was tested with the χ^2-based Cochran's statistic and the inconsistency index (I^2). Statistically significant heterogeneity was considered to be present when $P_{\text{heterogeneity}} < 0.05$ and $I^2 > 50\%$. If there was no statistical heterogeneity among studies ($I^2 < 50\%$ and $P_{\text{heterogeneity}} > 0.05$), the OR and 95%CI were estimated for each study in a fixed-effects model (FEM). Otherwise, a random effect model (REM) was used. A funnel plot was performed to look for evidence of publication bias; the funnel plot should be asymmetric when there is publication bias and symmetric in the case of no publication bias. Additionally, the publication bias was quantitatively estimated by Begg's and Egger's tests.

RESULTS

Study characteristics

Figure 1 summarizes the selection process of eligible studies. After a thorough literature search, 26 qualified publications[12-37] were included in this meta-analysis according to the inclusion criteria. Among these, 24 were included for the MTHFR C677T polymorphism and GC, and 11 were included for the MTHFR A1298C polymorphism and GC. The characteristics of the included studies, the variant genotypes and allele frequencies are listed in Table 1. Table 2 shows the available data on GCC and NCGC for MTHFR C677T and A1298C in detail. Additionally, data on intestinal and diffuse subtype GC for C677T were accessible in four studies (Table 3). According to the size of the heterogeneity, FEM or REM was adopted to analyze every comparison (Table 4).

Overall analysis

MTHFR C677T polymorphism and GC: Table 4 lists the main results of this meta-analysis. A total of 6266 cases and 8250 controls were identified for analysis of the association between the MTHFR C677T polymorphism and GC. The overall results showed that there was a significant association between C677T and GC [TT + CT *vs* CC: OR = 1.23 (1.08, 1.40), $P = 0.002$], and a T allele was associated with a 15.0% increased risk of GC compared to a C allele [T *vs* C: OR = 1.15 (1.06, 1.25), $P = 0.001$]. Similar results were obtained in the analysis for the additive model [TT *vs* CC: OR = 1.37 (1.15, 1.63), $P = 0.0005$] and the recessive model [TT *vs* CT + CC: OR = 1.17 (1.07, 1.28), $P = 0.0008$].

MTHFR A1298C polymorphism and GC: As shown in Table 4, 11 studies including a total of 2007 cases and 3679 controls were performed to analyze the relationship between the MTHFR A1298C polymorphism and GC. The risk for GC conferred by the MTHFR A1298C polymorphism did not reach significance under the four genetic models ($P > 0.05$).

Subgroup analysis

When stratifying the data by ethnicity, stronger significance between the MTHFR C677T polymorphism and GC was shown when restricted to eastern populations [TT + CT *vs* CC: OR = 1.26 (1.08, 1.47), $P = 0.003$; T *vs* C: OR = 1.21 (1.08, 1.34), $P = 0.0005$; TT *vs* CC: OR = 1.42 (1.15, 1.76), $P = 0.001$; TT *vs* CT + CC: OR = 1.22

Table 1 Characteristics of eligible studies included in the meta-analysis

Ref.	Country	Ethnicity	Sample size		C677T											A1298C										
					Genotypes distribution						Alleles frequency				*P* HWE	Genotypes distribution						Alleles frequency				*P* HWE
			Case	Control	Case			Control			Case		Control			Case			Control			Case		Control		
					CC	CT	TT	CC	CT	TT	C	T	C	T		AA	AC	CC	AA	AC	CC	A	C	A	C	
Gao *et al*[12]	China	Eastern	264	535	115	105	44	277	207	51	335	193	761	309	0.19											
Guo *et al*[13]	China	Eastern	97	114	22	48	27	32	57	25	92	102	121	107	0.97											
Saberi *et al*[14]	Iran	Eastern	405	780	198	172	35	422	308	50	578	242	1152	408	0.54											
Yang *et al*[15]	China	Eastern	139	165	44	80	15	62	75	28	168	110	197	131	0.5											
Cui *et al*[16]	South Korea	Eastern	2213	1700	778	1052	382	540	863	297	2608	1816	1943	1457	0.13											
De Re *et al*[17]	Italy	Western	48	96	12	23	13	37	46	13	47	49	120	72	0.73	24	19	5	33	54	9	67	29	120	72	0.08
Galvan-Portillo *et al*[18]	Mexico	Western	248	478	37	132	79	89	217	172	216	290	395	561	0.17											
Gotze *et al*[19]	Germany	Western	106	106	46	45	12	41	49	16	137	69	131	81	0.74											
Zeybek *et al*[20]	Turkey	Eastern	35	144	18	12	5	64	65	15	48	22	193	95	0.76											
Vollset *et al*[21]	Europea[1]	Western	247	631	109	104	32	248	277	94	322	168	773	465	0.27	103	116	25	315	246	53	322	166	876	352	0.61
Mu *et al*[22]	China	Eastern	196	397	50	106	38	135	199	57	206	182	469	313	0.23	147	49	0	275	112	7	343	49	662	126	0.25
Zhang *et al*[23]	Poland	Western	305	427	146	116	33	185	178	36	408	182	548	250	0.48	135	125	31	180	179	41	395	187	539	261	0.72
Boccia *et al*[24]	Italy	Western	102	254	29	51	22	98	115	41	109	95	311	197	0.43	50	43	9	125	107	22	143	61	357	151	0.89
Fu *et al*[25]	China	Eastern	169	169												96	73	0	125	44	0	265	73	294	44	0.12
Graziano *et al*[26]	Italy	Western	162	164	34	86	42	67	68	29	154	126	202	126	0.10											
Li *et al*[27]	China	Eastern	170	140	61	78	31	67	56	17	200	140	190	90	0.32	126	42	2	97	41	2	294	46	235	45	0.32
Weng *et al*[28]	China	Eastern	38	34	14	19	5	15	11	8	47	29	41	27	0.06	26	12	0	22	11	1	64	12	55	13	0.79
Kim *et al*[29]	South Korea	Eastern	133	445	42	64	27	143	239	63	148	118	525	365	0.02[2]	98	34	1	308	129	8	230	36	745	145	0.19
Si *et al*[30]	China	Eastern	122	101	58	48	16	49	43	9	164	80	141	61	0.92	73	44	5	58	38	5	190	54	154	48	0.70
Sarbia *et al*[31]	Germany	Western	332	255	138	153	41	107	115	33	429	235	329	181	0.81											
Bi *et al*[32]	China	Eastern	309	188	139	150	20	97	76	15	428	190	270	106	0.99											
Wang *et al*[33]	China	Eastern	129	315	25	45	59	74	143	98	95	163	291	339	0.12											
Stolzenberg-Solomon *et al*[34]	China	Eastern	90	398	17	36	37	65	209	124	70	110	339	457	0.14	69	21	0	294	104	0	159	21	692	104	0.003[2]
Miao *et al*[35]	China	Eastern	217	468	47	107	63	151	217	100	201	233	519	417	0.18	150	64	3	324	139	5	364	70	787	149	0.02[2]
Gao *et al*[36]	China	Eastern	107	200	22	61	24	63	99	38	105	109	225	175	0.93											
Shen *et al*[37]	China	Eastern	187	166	55	90	42	60	80	26	200	174	200	132	0.96	130	55	2	111	50	5	315	59	272	60	0.83

[1]Study involving Denmark, France, Greece, Germany, Italy, the Netherlands, Norway, Spain, Sweden, and United Kingdom; [2]Studies with the controls not in Hardy-Weinberg equilibrium (HWE).

(1.09, 1.35), $P = 0.0003$], although the result for western populations was not significant (Table 4). With regard to the A1298C polymorphism, the results showed that there was a significant association between the A1298C polymorphism and a decreased risk of GC in eastern populations in the additive and recessive models [CC *vs* AA: OR = 0.44 (0.21, 0.93), $P = 0.03$; CC *vs* AC + AA: OR = 0.46 (0.22, 0.96), $P = 0.04$] (Table 4).

Stratification analyses were performed to consider tumor sites. The results suggested that the MTHFR C677T polymorphism was significantly associated with an increased GC risk for both GCC [TT *vs* CT + CC: OR = 1.38 (1.15, 1.67), $P = 0.0006$] and NCGC [TT + CT *vs* CC: OR = 1.38 (1.18, 1.63), $P < 0.0001$; T *vs* C: OR = 1.24 (1.10, 1.40), $P = 0.0003$; TT *vs* CC: OR = 1.44 (1.09, 1.91), $P = 0.01$] in the whole population. When further analyzed by ethnicity, similar positive results were found only in eastern but not in western populations. We also obtained no significant results for the association between A1298C and both GCC and NCGC (Table 4).

In view of the Lauren classification, we divided the studies into two subgroups. The results showed that the MTHFR C677T polymorphism was significantly associated with an increased risk for intestinal-type GC [TT *vs* CC: OR = 1.88 (1.22, 2.89), $P = 0.004$; TT *vs* CT + CC: OR = 1.45 (1.07, 1.95), $P = 0.02$] in the whole population. When further

Table 2 Distribution of MTHFR C677T and A1298C genotypes and alleles frequency in gastric cardia cancer, non-cardia cancer and controls

Ref.	Sample size			Genotypes distribution									Alleles frequency					
	Cardia	Non-cardia	Control	Cardia			Non-cardia			Control			Cardia		Non-cardia		Control	
MTHFR C677T polymorphism				CC	CT	TT	CC	CT	TT	CC	CT	TT	C	T	C	T	C	T
Saberi *et al*[14]	152	210	780	77	60	15	99	98	13	422	308	50	214	90	296	124	1152	408
Gotze *et al*[19]	27	76	106	13	13	1	33	32	11	41	49	16	39	15	98	54	131	81
Graziano *et al*[26]	43	119	164	7	25	11	27	61	31	67	68	29	39	47	115	123	202	126
Weng *et al*[28]	NA	38	34		NA		14	19	5	15	11	8	NA		47	29	41	27
Sarbia *et al*[31]	119	213	255	65	45	9	73	108	32	107	115	33	175	63	254	172	329	181
Si *et al*[30]	29	93	101	21	7	1	37	41	15	49	43	9	49	9	115	71	141	61
Bi *et al*[32]	155	154	188	74	73	8	65	77	12	97	76	15	211	89	207	101	270	106
Wang *et al*[33]	129	NA	315	25	45	59		NA		74	143	98	95	163	NA		291	339
Stolzenberg-Solomon *et al*[34]	90	NA	398	17	36	37		NA		65	209	124	70	110	NA		339	457
Miao *et al*[35]	217	NA	468	47	107	63		NA		151	217	100	201	233	NA		519	417
Shen *et al*[37]	82	105	166	22	38	22	33	52	20	60	80	26	82	82	118	92	200	132
MTHFR A1298C polymorphism				AA	AC	CC	AA	AC	CC	AA	AC	CC	A	C	A	C	A	C
Weng *et al*[28]	NA	38	34		NA		26	12	0	22	11	1	NA		64	12	55	13
Si *et al*[30]	29	93	101	15	12	2	58	32	3	58	38	5	42	16	148	38	154	48
Shen *et al*[37]	82	105	166	64	17	1	66	38	1	111	50	5	145	19	170	40	272	60

NA: Not available.

Table 3 Distribution of MTHFR C677T and A1298C genotypes and alleles frequency in intestinal, diffuse gastric cancer and controls

Ref.	Sample size			Genotypes distribution									Alleles frequency					
	Intestinal	Diffuse	Control	Intestinal			Diffuse			Control			Intestinal		Diffuse		Control	
				CC	CT	TT	CC	CT	TT	CC	CT	TT	C	T	C	T	C	T
MTHFR C677T polymorphism																		
Saberi *et al*[14]	142	80	780	69	59	14	39	34	7	422	308	50	197	87	112	48	1152	408
Galvan-Portillo *et al*[18]	88	152	454	50		38	113		39	291		163	NA		NA		NA	
Gotze *et al*[19]	53	37	106	21	24	8	18	15	4	41	49	16	66	40	51	23	131	81
Graziano *et al*[26]	91	71	164	19	47	25	15	39	17	67	68	29	85	97	69	73	202	126

NA: Not available.

analyzed by ethnicity, similar positive results were found only in western populations but not in eastern populations. No significant relationship was found between the MTHFR C677T polymorphism and diffuse-type GC (Table 4).

Relationships between GCC and NCGC, and intestinal-type and diffuse-type GC

When comparing GCC with NCGC, no significant results were observed in any of the four models for C677T or A1298C (Table 5), and ORs of 1.71 (95%CI: 1.13-2.59, $P = 0.01$) and 1.60 (95%CI: 1.09-2.35, $P = 0.02$) were found in the TT *vs* CC model in western populations and total populations, respectively, when comparing intestinal-type with diffuse-type GC for C677T. None of the other models produced significant results for eastern, western or overall populations (Table 5).

Sensitivity analysis and publication bias evaluation

A sensitivity analysis was performed by excluding one study each time to reflect the influence of the individual data set on the ORs; the analysis did not alter the pattern of the results (data not shown), which confirmed the stability of the above results. The funnel plot (data not shown) provided no evidence of publication bias. Consistent results were drawn from Begg's and Egger's tests.

DISCUSSION

Regarding the MTHFR C677T and A1298C polymorphisms and their association with GC, definite conclusions cannot be drawn. Therefore, we performed a meta-analysis to estimate the relationships between the two SNPs in the *MTHFR* gene and the risk of GC.

In the present meta-analysis, the overall analysis suggested that MTHFR 677TT and CT genotype carriers had a higher risk of developing GC; in addition, an elevated risk of GC was also found among the MTHFR 677T allele carriers. It is well known that individuals who are MTHFR 677T carriers have reduced MTHFR activity[10], and the low enzyme activity of MTHFR C677T variant genotypes is associated with DNA hypomethylation, which may induce genomic instability and thereby affect the expression of oncogenes or tumor suppressor genes, leading to the development of malignancies[45,46]. No significant association was found between the MTH-

Table 4 Comparisons of MTHFR C677T and A1298C polymorphisms for whole and stratified analysis

	n	Case/control	OR (95%CI)	*P*	$P_{Heterogeneity}$	OR (95%CI)	*P*	$P_{Heterogeneity}$	OR (95%CI)	*P*	$P_{Heterogeneity}$	OR (95%CI)	*P*	$P_{Heterogeneity}$
			(TT + CT)/CC			T/C			TT/CC			TT/(CT + CC)		
C677T														
Total	24	6266/8250	1.23 (1.08, 1.40)	0.002	< 0.0001	1.15 (1.06, 1.25)	0.001	0.0004	1.37 (1.15, 1.63)	0.0005	0.0005	1.17 (1.07, 1.28)	0.0008	0.01
Eastern	16	4716/5839	1.26 (1.08, 1.47)	0.003	0.002	1.21 (1.08, 1.34)	0.0005	0.002	1.42 (1.15, 1.76)	0.001	0.005	1.22 (1.09, 1.35)	0.0003	0.03
Western	8	1550/2411	1.20 (0.92, 1.56)	0.180	0.002	1.05 (0.91, 1.21)	0.53	0.05	1.28 (0.91, 1.82)	0.16	0.009	1.05 (0.88, 1.25)	0.55	0.11
Tumor site														
Cardia														
Total	10	1043/2941	1.09 (0.81, 1.48)	0.560	0.0005	1.16 (0.95, 1.41)	0.14	0.002	1.28 (0.85, 1.95)	0.24	0.003	1.38 (1.15, 1.67)	0.0006	0.05
Eastern	7	854/2416	1.16 (0.89, 1.52)	0.280	0.05	1.24 (1.04, 1.48)	0.02	0.06	1.57(1.23, 2.00)	0.0003	0.16	1.53 (1.25, 1.87)	< 0.0001	0.31
Western	3	189/525	1.09 (0.38, 3.12)	0.870	0.001	1.00 (0.52, 1.91)	1.00	0.004	0.78 (0.15, 4.16)	0.77	0.003	0.73 (0.27, 1.99)	0.54	0.07
Non-cardia														
Total	8	1008/1794	1.38 (1.18, 1.63)	< 0.0001	0.42	1.24 (1.10, 1.40)	0.0003	0.42	1.44 (1.09, 1.91)	0.01	0.31	1.19 (0.93, 1.52)	0.16	0.61
Eastern	5	600/1269	1.35 (1.10, 1.66)	0.004	0.99	1.21 (1.04, 1.41)	0.01	0.87	1.30 (0.87, 1.95)	0.20	0.48	1.10 (0.79, 1.55)	0.57	0.44
Western	3	408/525	1.41 (0.83, 2.38)	0.400	0.03	1.26 (0.91, 1.76)	0.16	0.06	1.55 (0.86, 2.78)	0.14	0.12	1.29 (0.91, 1.83)	0.16	0.53
Lauren's classification														
Intestinal														
Total	4	374/1504	1.46 (0.86, 2.47)	0.160	0.05	1.33 (0.97, 1.84)	0.08	0.10	1.88 (1.22, 2.89)	0.004	0.19	1.45 (1.07, 1.95)	0.02	0.76
Eastern	1	142/780	1.25 (0.87,1.87)	0.230	NA	1.25 (0.95, 1.64)	0.12	NA	1.71 (0.90, 3.26)	0.10	NA	1.60 (0.86, 2.97)	0.14	NA
Western	3	232/724	1.61 (0.60,4.29)	0.340	0.03	1.37 (0.74, 2.52)	0.32	0.04	2.01 (1.13, 3.59)	0.02	0.07	1.41 (1.00, 1.98)	0.05	0.58
Diffuse														
Total	4	340/1504	1.31 (0.66,2.58)	0.440	0.03	1.19 (0.78, 1.82)	0.42	0.06	1.46 (0.66, 3.21)	0.35	0.13	0.94 (0.56, 1.58)	0.82	0.09
Eastern	1	80/780	1.24 (0.78,1.96)	0.360	NA	1.21 (0.85, 1.73)	0.29	NA	1.51 (0.64, 3.57)	0.34	NA	1.40 (0.61, 3.20)	0.80	NA
Western	3	260/724	1.33 (0.35,5.01)	0.670	0.008	1.14 (0.50, 2.60)	0.76	0.02	1.31 (0.29, 5.83)	0.72	0.04	0.85 (0.46, 1.55)	0.59	0.1
A1298C			(CC + AC)/AA			C/A			CC/AA			CC/(AC + AA)		
Total	11	2007/3679	0.98 (0.79, 1.21)	0.840	0.008	0.97 (0.83, 1.14)	0.73	0.03	0.95 (0.71, 1.28)	0.76	0.60	0.95 (0.72, 1.27)	0.75	0.78
Eastern	7	1015/1452	0.96 (0.72, 1.29)	0.790	0.02	0.93 (0.73, 1.19)	0.56	0.04	0.44 (0.21, 0.93)	0.03	0.84	0.46 (0.22, 0.96)	0.04	0
Western	4	702/1408	1.00 (0.71, 1.41)	0.990	0.04	1.07 (0.93, 1.23)	0.36	0.15	1.13 (0.82, 1.57)	0.46	0.69	1.11 (0.81, 1.51)	0.53	0.98
Tumor site														
Cardia														
Total	2	111/267	0.80 (0.37, 1.74)	0.240	0.13	0.83 (0.41, 1.69)	0.61	0.10	0.76 (0.21, 2.83)	0.69	0.28	0.80 (0.22, 2.93)	0.73	0.36
Eastern	2	111/267	0.80 (0.37, 1.74)	0.240	0.13	0.83 (0.41, 1.69)	0.61	0.10	0.76 (0.21, 2.83)	0.69	0.28	0.80 (0.22, 2.93)	0.73	0.36
Western	0	0	NA			NA			NA			NA		
Non-cardia														
Total	3	236/301	0.98 (0.69, 1.41)	0.930	0.59	0.93 (0.68, 1.26)	0.62	0.69	0.45 (0.15, 1.40)	0.17	0.86	0.46 (0.15, 1.40)	0.17	0.82
Eastern	3	236/301	0.98 (0.69, 1.41)	0.930	0.59	0.93 (0.68, 1.26)	0.62	0.69	0.45 (0.15, 1.40)	0.17	0.86	0.46 (0.15, 1.40)	0.17	0.82
Western	0	0	NA			NA			NA			NA		

NA: Not available; $P_{Heterogeneity}$: *P* value of *Q* test for heterogeneity test.

Table 5 Comparisons of MTHFR C677T and A1298C polymorphisms for gastric cardia and non-cardia cancers, intestinal-type and diffuse-type gastric cancers

	n	Case/control	OR (95%CI)	*P*	$P_{Heterogeneity}$	OR (95%CI)	*P*	$P_{Heterogeneity}$	OR (95%CI)	*P*	$P_{Heterogeneity}$	OR (95%CI)	*P*	$P_{Heterogeneity}$
C677T			(TT + CT)/CC			T/C			TT/CC			TT/(CT + CC)		
Cardia and non-cardia														
Total	7	607/970	0.74 (0.51, 1.08)	0.12	0.01	0.80 (0.59, 1.09)	0.2	0.002	0.82 (0.49, 1.38)	0.46	0.01	0.71 (0.36, 1.39)	0.31	0.06
Eastern	4	418/562	0.76 (0.47, 1.21)	0.24	0.04	0.85 (0.56, 1.27)	0.4	0.01	1.04 (0.52, 2.06)	0.91	0.05	0.89 (0.39, 2.03)	0.78	0.10
Western	3	189/408	0.75 (0.35, 1.61)	0.46	0.04	0.73 (0.45, 1.20)	0.2	0.05	0.59 (0.35, 1.00)	0.05	0.08	0.52 (0.17, 1.59)	0.25	0.26
Intestinal-type and diffuse-type														
Total	4	374/340	1.09 (0.74, 1.62)	0.67	0.76	1.10 (0.84, 1.45)	0.5	0.78	1.60 (1.09, 2.35)	0.02	0.87	1.24 (0.68, 2.27)	0.48	0.50
Eastern	1	142/80	1.01 (0.58, 1.74)	0.98	NA	1.03 (0.68, 1.57)	0.9	NA	1.14 (0.44, 2.95)	0.79	NA	1.13 (0.42, 3.04)	0.81	NA
Western	3	232/260	1.19 (0.68, 2.09)	0.55	0.54	1.16 (0.81, 1.66)	0.4	0.57	1.71 (1.13, 2.59)	0.01	0.64	1.32 (0.62, 2.80)	0.48	0.41
A1298C			(CC + AC)/AA			C/A			CC/AA			CC/(AC + AA)		
Cardia and non-cardia														
Total	2	111/198	0.83 (0.26, 2.64)	0.75	0.03	0.90 (0.34, 2.34)	0.8	0.03	1.87 (0.39, 9.03)	0.44	0.59	1.85 (0.39, 8.69)	0.43	0.75
Eastern	2	111/199	0.83 (0.26, 2.64)	0.75	0.03	0.90 (0.34, 2.34)	0.8	0.03	1.87 (0.39, 9.03)	0.44	0.59	1.85 (0.39, 8.69)	0.43	0.75
Western	0	0	NA			NA			NA			NA		

NA: Not available; $P_{Heterogeneity}$: *P* value of *Q* test for heterogeneity test.

FR A1298C polymorphism and overall GC risk; a possible explanation for which could be that the reduction of MTHFR functional activity caused by the A1298C mutation is significantly less than that caused by the C677T mutation[9].

In subgroup analyses stratified by the ethnicity, gastric tumor site and Lauren classification, we found that the MTHFR C677T polymorphism was associated with susceptibility to both GCC and NCGC in eastern populations compared with controls. No positive association was found between the MTHFR C677T polymorphism and the risk of intestinal or diffuse types of GC compared with controls. With regard to the A1298C polymorphism, we found that the CC genotype conferred protection against GC in eastern but not in Western populations; however, the inconsistent results among Western and Eastern populations are difficult to explain. Moreover, irrespective of comparison with controls or diffuse-type GC, a positive association was found that the C677TT polymorphism increased the risk of intestinal-type GC in the whole population and in the western population. No significant difference was found between GCC and NCGC. Because a small sample size was included, this conclusion remains to be confirmed. To the best of our knowledge, the distribution of the *MTHFR* polymorphism differs among various ethnic populations[47], which may have led to the different results for eastern and western populations.

Although several related meta-analyses have been published previously[38-44], our current research still has some advantages. First, because it involved 26 studies conforming to HWE and provided 6390/8515 cases/controls, our meta-analysis included a larger number of studies than the previous studies, and the results are more reliable. Second, our study is the first to include stratification according to tumor site and Lauren classification.

There was a certain degree of heterogeneity among the studies assessed here, which may be attributed to design quality, sample size, noncomparable measures of genotyping, and variation of the covariate. To clarify the sources of heterogeneity, we conducted a sensitivity analysis, and this analysis confirmed the stability of the null association between *MTHFR* polymorphisms and GC after excluding any one study at a time.

No significant publication bias was found herein given the symmetry shown in the funnel plots, and consistent results were drawn from Begg's and Egger's tests (data not shown). Nevertheless, unpublished data from conference abstracts and dissertations and unpublished pharmaceutical company data were not extracted, which could introduce a distinct possibility of publication bias. Moreover, we followed the inclusion and exclusion criteria strictly to reduce selection bias. In addition, the test of HWE for the distribution of the genotypes in the control groups suggested that there were no individuals with significantly aberrant genetic backgrounds among the participants.

Nevertheless, this meta-analysis had several limitations that may have affected the conclusions. First, we selected only the MTHFR C677T and A1298C polymorphisms

because these were the most extensively studied polymorphisms, although several other SNPs in the *MTHFR* gene have been identified. Meta-analyses that investigate the association of other polymorphisms in the *MTHFR* gene with GC should be performed in the future. Second, study design, small sample size and environmental factors may have affected the results; many studies did not use an appropriate design or neglected to consider important environmental factors. Third, the results drawn from subgroup analyses might be limited because of the small sample size. Moreover, it was difficult to obtain full papers published in various languages; we included studies published only in English and Chinese.

In summary, data from our meta-analysis support that the MTHFR C677T polymorphism increases the risk of developing GC in the general population, as well as the risk of GCC and NCGC in eastern populations and intestinal-type GC in western populations. The A1298C polymorphism may be a protective factor against GC in eastern populations. GC is a disease resulting from complex interactions between genes and the environment. Therefore, further well-designed studies with larger sample sizes should be performed to assess other genetic and environmental factors in the development of GC.

COMMENTS

Background

Currently, the incidence of gastric cancer (GC) has decreased worldwide, but it remains the fourth most common cancer diagnosis in men, and the fifth in women, and the second leading cause of cancer-related death. Methylenetetrahydrofolate reductase (MTHFR) encodes a 77-kDa protein that plays a key role in DNA methylation. Many studies have explored the association between *MTHFR* polymorphisms and GC risk, but the results remain either controversial or inconclusive. Consequently, the authors performed a meta-analysis to clarify the role of *MTHFR* polymorphisms in GC susceptibility among the eligible studies.

Research frontiers

Until now, many studies have reported associations of *MTHFR* polymorphisms with susceptibility to GC; however, the results have been inconsistent and inconclusive.

Innovations and breakthroughs

This meta-analysis indicates that the MTHFR C677T polymorphism is a risk factor in GC and that the A1298C polymorphism may be a protective factor against GC in eastern populations. Moreover, this study is the first to include stratification according to tumor site and Lauren classification.

Applications

This meta-analysis showed that the C677T and A1298C polymorphisms of the *MTHFR* gene could alter susceptibility to GC. The findings may provide valuable information about the etiology of GC for both researchers and clinicians.

Terminology

MTHFR encodes a 77-kDa protein that plays a key role in folate metabolism by irreversibly catalyzing the reduction of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the predominant circulatory form of folate, which serves as both a cofactor and substrate for the regeneration of methionine. The latter leads to the production of *S*-adenosylmethionine (SAM), the universal methyl donor in humans for DNA methylation. Reduced enzyme activity may result in lower levels of SAM and an increased risk of cancer, including GC, as a consequence of gene hypomethylation.

Peer review

This meta-analysis was a well-written and well-conducted study that evaluated the association of *MTHFR* polymorphisms with susceptibility to gastric cancer. It had a large sample size, which allowed consistent conclusions in relation to the general population. Additionally, this study is the first to include stratification according to gastric cancer location and histological subtype. It is important to review these relevant reports systematically.

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P- Reviewer: Jonaitis L, Santos JS, Zhang SN **S- Editor**: Gou SX
L- Editor: Kerr C **E- Editor**: Zhang DN

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DOI: 10.3748/wjg.v20.i32.11439

World J Gastroenterol 2014 August 28; 20(32): 11439-11442
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

First report of colonoscopic closure of a gastrocolocutaneous PEG migration with over-the-scope-clip-system

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Received: January 26, 2014 Revised: March 11, 2014
Accepted: April 28, 2014
Published online: August 28, 2014

Abstract

Percutaneous endoscopic gastrostomy (PEG) is a common practice for long-term nutrition of patients who are unable to take oral food. We report of an 85-year old man with a history of recurrent larynx carcinoma and hemicolectomy many years ago due to unknown reason. Laryngectomy was indicated. Preoperatively a PEG was inserted endoscopically after an abdominal ultrasonography without abnormal findings. Few months after PEG insertion, the patient was evaluated for diarrhea and insufficient feeding without signs of infection or peritonism. An upper endoscopy and computed tomography scan confirmed a buried bumper syndrome with migration of the PEG tube into the colon as a rare complication. He underwent successful colonoscopic removal of the internal bumper and closure of the colonic orifice of the fistula with the over-the-scope-clip system (OTSC). OTSC is an endoscopic device for treatment of bleeding, perforation, leak and fistula in the gastrointestinal tract. To the best of our knowledge, this is the first report of the use of OTSC for colonoscopic closure of a gastrocolocutaneous fistula due to a buried bumper syndrome with transcolonic PEG tube migration.

Key words: Over-the-scope-clip; Over-the-scope-clip system; Enterocutaneous fistula; Colon; Buried bumper; Percutaneous endoscopic gastrostomy; Complication; Migration

Core tip: We present a case of buried bumper syndrome with percutaneous endoscopic gastrostomy tube migration into the transverse colon that was treated endoscopically using the over-the-scope-clip (OTSC) system for closure. OTSC is a new endoscopic device for treatment of bleeding, perforations, leaks and fistulae in the gastrointestinal tract.

Bertolini R, Meyenberger C, Sulz MC. First report of colonoscopic closure of a gastrocolocutaneous PEG migration with over-the-scope-clip-system. *World J Gastroenterol* 2014; 20(32): 11439-11442 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11439.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11439

INTRODUCTION

Percutaneous endoscopic gastrostomy (PEG) tubes are widely used for long-term enteral nutrition for patients who are unable to take oral food, *e.g.*, after head and neck surgery. PEG tube insertion is associated with some potential complications although it is generally considered to be safe[1].

The over-the-scope-clip (OTSC) system (Ovesco Endoscopy AG, Tübingen, Germany) is a new endoscopic device suitable to close perforations[2,3], post-surgical fistulae[3,4] or resection of submucosal tumors[5]. It can also be used as hemostatic tool in gastrointestinal bleeding[6] and for esophageal stent fixation[5]. To the best of our knowledge, in literature there are only case reports or

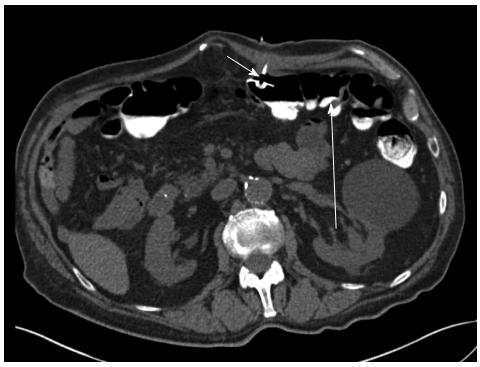

Figure 1 Computed tomography of the abdomen confirmed a buried bumper syndrome with displacement of the internal bumper (short arrow) into the transverse colon (long arrow).

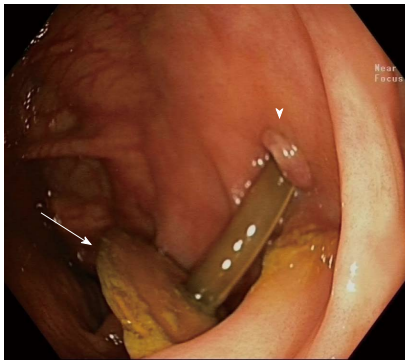

Figure 2 Colonoscopy also revealed the internal bumper (arrow) in the transverse colon. The fistula (arrowhead) measured 3-4 mm in diameter.

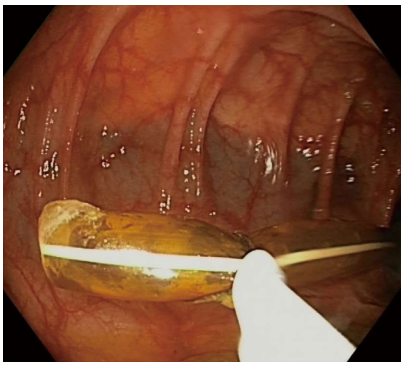

Figure 3 Removal of the internal bumper with a polypectomy snare.

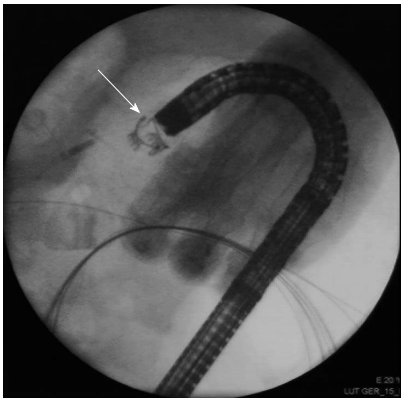

Figure 4 Over-the-scope-clip system device (arrow) is being deployed on top of the fistula.

small case series regarding the efficacy and safety[2-8], so far randomized controlled trials are not available.

A rare complication of PEG insertion is a secondary PEG migration from the stomach into the colon[1,9,10]. In our case the migrated PEG was removed via colonoscopy and the fistula was closed with an OTSC.

CASE REPORT

An 85-year-old man was hospitalized with a recurrent larynx carcinoma and pharyngotracheal fistula after radiotherapy and laser cordectomy. Laryngectomy was indicated. To avoid oral intake, a PEG was planned after an abdominal ultrasonography without abnormal findings. The patient had undergone a laparotomy with right-sided hemicolectomy many years ago. Based on the available records we could not find out the reason for that hemicolectomy.

The endoscopic transillumination prior to gastric puncture was not easy to perform. A PEG tube (15 Charrière; Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany) was inserted and the internal bumper placement could be confirmed endoscopically. After one week, peristomal putrid leakage as well as limited in and out movement was reported. The clinical examination revealed neither signs of peritonism nor relevant local infection. The tube could be flushed easily. A plain radiograph and an ultrasonography of the abdomen were normal. In order to prevent recurrent regurgitation two weeks after insertion, a jejunal extension was performed endoscopically, the internal bumper was still correctly placed. Five months later, again putrid leakage beside the PEG tube was observed and it was difficult to flush the gastric port whereas the jejunal port worked properly. Initially the tube could be moved toughly, but then easily, possibly due to a beginning buried bumper syndrome. Ten months after PEG tube insertion the patient was re-evaluated for therapy-refractory diarrhea and insufficient feeding. The upper endoscopy revealed a buried bumper syndrome. The internal bumper was covered with scarf tissue, only a small fistula was present. We carefully inserted an atraumatic guide wire into the gastrostomy tube without resistance, but it was not endoscopically visible. Computed tomography confirmed a buried bumper syndrome with displacement of the internal bumper into the transverse colon (Figure 1). A colonoscopy also revealed the internal bumper in the transverse colon, the colocutaneous fistula measured about 3-4 mm in diameter (Figure 2). The internal bumper was removed with a polypectomy snare (Figure 3). A 1TQ-160 gastroscope (Olympus, Tokyo, Japan) was re-inserted with the mounted and loaded OTSC device (size 12/6 mm type a 10 mm), that was released under suction (Figures 4 and 5). Despite air insufflation no air leaked through the skin. The patient

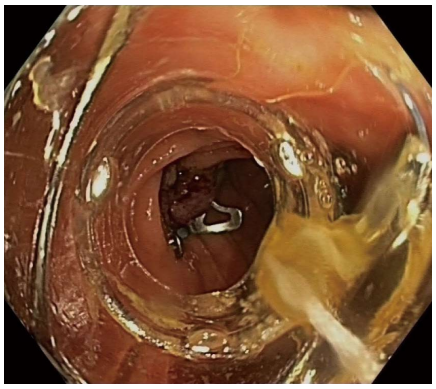

Figure 5 Deployed over-the-scope-clip system device in transcolonic fistula.

underwent a new PEG and continued PEG feeding. Diarrhea stopped.

DISCUSSION

The OTSC system shows a great variety of treatment options in the upper and lower GI tract. OTSC application within the colon has been described as successful tool to close anastomotic dehiscence, post-surgical fistulae[4], colovesicular fistulae with diverticulosis, colocutaneous fistulae[2], endoscopic R1 resection of a adenocarcinoma of the sigma[3], perforation after polypectomy[6], endoscopic submucosal dissection[7] as well as acute perforation with gastrointestinal bleeding of the colonic wall one day after PEG[3].

To the best of our knowledge, this is the first report of the use of OTSC for colonoscopic closure of a gastrocolocutaneous fistula due to a buried bumper syndrome with transcolonic PEG tube migration. We decided to close the colonic fistula orifice after colonoscopic removal of the PEG tube because the colonic fistula orifice was greater than the gastric one and the tissue of the colonic wall was easy to suck. Recently, Murino *et al*[11] also reported a case of gastrocolic fistula secondary to transcolonic PEG tube migration. However, they used the OTSC to close the gastric orifice. Mönkemüller *et al*[12] successfully closed a gastrocolic fistula in a severely malnourished patient with an OTSC application at the gastrojejunal anastomosis after Billroth Ⅱ.

After abdominal surgery, in our case laparotomy and right-sided hemicolectomy, the transverse colon can be displaced over the anterior gastric wall. In those settings, the colon can be injured and a gastrocolocutaneous fistula can occur during PEG placement. This potential complication of PEG tube insertion is very rare[1,9,10]. Transcolonic displacement of the PEG tube can present with diarrhea, fecal discharge or rarely without symptoms. Some patients need surgery due to peritonitis or abdominal sepsis[13]. However, a conservative procedure by removal of the tube or colonoscopic clip application can also be successful[14,15].

Some important issues need to be addressed to prevent this complication. Pre- and peri-interventional imaging examinations are essential for adequate prevention. In our case, we performed a pre-interventional abdominal ultrasonography, but this imaging modality is not adequately suitable to detect a colonic interposition. The most important issue is that gastric insufflation and transillumination should be adequate. In this case, transillumination was difficult to obtain. Furthermore, an abdominal fingerprint impression should indent the gastric wall, and a sudden escape of gas or stool using a pilot needle not visible endoscopically indicates the puncture of another structure[1]. Tominaga *et al*[9] recommend the use of a colonoscope to facilitate the colonic displacement after abdominal surgery.

In conclusion, we report the first use of OTSC for colonoscopic closure of a gastrocolocutaneous fistula due to a buried bumper syndrome with transcolonic PEG tube migration.

COMMENTS

Case characteristics

An 85-year-old man with a history of percutaneous endoscopic gastrostomy few months ago and right-sided hemicolectomy many years ago presented with diarrhea and insufficient feeding.

Clinical diagnosis

Neither signs of peritonism nor fever.

Differential diagnosis

Infection, colitis, malabsorption syndrome, percutaneous endoscopic gastrostomy (PEG) migration, perforation.

Laboratory diagnosis

Hemoglobin 100 g/L, white blood cell 5 g/L, C-reactive protein 53 mg/L.

Imaging diagnosis

Computed tomography confirmed buried bumper syndrome with displacement, localizing the position of the internal bumper in the transverse colon. No free fluid collection or gas intraabdominal.

Endoscopic diagnosis

Colonoscopy revealed the internal bumper within the transverse colon.

Treatment

The PEG tube was removed with a polypectomy snare and the gastrocolocutaneus fistula was closed colonoscopically with an over-the-scope-clip (OVESCO Endoscopy AG, Tübingen, Germany).

Related reports

Over-the-scope-clip is a new endoscopic device for closure of lesions in the GI tract.

Experiences and lessons

This report used the over-the-scope-clip system for colonoscopic treatment of a gastrocolocutaneous fistula in a patient with buried bumper syndrome and secondary PEG migration. Important issues are discussed to prevent this very rare complication of PEG tube insertion.

Peer review

This is an interesting case report that support the utilization of over-the-scope-clip system (OTSC) device for coloscopic closure of a gastrocolocutaneous fistula due to a buried bumper syndrome with transcolonic PEG tube migration. In my opinion this is a very original and useful application of OTSC device.

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DOI: 10.3748/wjg.v20.i32.11443

World J Gastroenterol 2014 August 28; 20(32): 11443-11450
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Intestinal pseudo-obstruction in patients with systemic lupus erythematosus: A real diagnostic challenge

Carlos Alberto García López, Fernando Laredo-Sánchez, José Malagón-Rangel, Miguel G Flores-Padilla, Haiko Nellen-Hummel

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Author contributions: García López CA, Laredo-Sánchez F, Malagón-Rangel J and Flores-Padilla MG had contact with the patients and collected their information; García López CA and Laredo-Sánchez F designed the idea and prepared the figures for the manuscript; Malagón-Rangel J synthesized the text; Flores-Padilla MG reviewed and revised the manuscript; Nellen-Hummel H reviewed and revised the final manuscript; García López CA was responsible for writing the manuscript.

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Received: January 5, 2014 Revised: March 11, 2014
Accepted: April 15, 2014
Published online: August 28, 2014

Key words: Intestinal pseudo-obstruction; Systemic lupus erythematosus; Uretero-hydronephrosis; Urinary tract infection; Diagnostic challenge

Core tip: We present a summary of the most important clinical points in four clinical cases of intestinal pseudo-obstruction secondary to systemic lupus erythematosus using a suggested approach in order to reach a prompt diagnosis due to this pathology presenting a picture of acute abdomen. It is a case series of Mexican patients and, to the best of our knowledge in this particular presentation, is the first report in the international medical literature.

García López CA, Laredo-Sánchez F, Malagón-Rangel J, Flores-Padilla MG, Nellen-Hummel H. Intestinal pseudo-obstruction in patients with systemic lupus erythematosus: A real diagnostic challenge. *World J Gastroenterol* 2014; 20(32): 11443-11450 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11443.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11443

Abstract

Intestinal pseudo-obstruction secondary to systemic lupus erythematosus (SLE) is a rare syndrome described in recent decades. There are slightly over 30 published cases in the English language literature, primarily associated with renal and hematological disease activity. Its presentation and evolution are a diagnostic challenge for the clinician. We present four cases of intestinal pseudo-obstruction due to lupus in young Mexican females. One patient had a previous diagnosis of SLE and all presented with a urinary tract infection of varying degrees of severity during their evolution. We consider that recognition of the disease is of vital importance because it allows for establishing appropriate management, leading to a better prognosis and avoiding unnecessary surgery and complications.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a disease caused by an aberrant immune response[1]. Its manifestations range from positive serology to one (or multiple) organ dysfunction and even death. Its course is unpredictable and involves periods of remissions and relapses[1-3]. Gastrointestinal (GI) manifestations may occur in up to 50% of patients with SLE[4,5] with the majority being mild, such as secondary effects to medication[6-8]. However, the clinical spectrum reported in the literature shows that presentations are usually associated with activity at another level[4,5,8-10] and can be aggressive, placing the life of the patient at risk[4,5,10]. Intestinal pseudo-obstruction is characterized by an ineffective propulsion of the intestine without obvious mechanical cause[6,10,11]. Its association with SLE is described infrequently. The significance

Table 1 Characteristics of patients with intestinal pseudo-obstruction due to systemic lupus erythematosus at the time of diagnosis

	Case 1	Case 2	Case 3	Case 4
General information				
Age (yr)	27	23	25	37
Time of diagnosis of SLE	0 mo/yr	0 mo/yr	0 mo/yr	12 yr
SLEDAI	27	11	15	10
Clinical signs and symptoms				
Weight loss (kg)	10	15	8	NR
Seizures/amaurosis	+	-	-	-
Fever	+	+	+	+
Serositis (pleural effusion)	+	+	+	+
Ascites	+	-	-	-
Dysuria	+	+	+	-
Sepsis	+	+	-	-
Paraclinical tests				
Uretero-hydronephrosis (CT)	+	+	+	+
Esophagitis (endoscopy)	+	+	NR	NR
Hypomotility (manometry)	+	+	NR	NR
Laboratory tests				
ANA (IU/mL)	2	1.1	3.4	NR
Anti-ds-DNA (IU/mL)	29	34	40	NR
C3 (mg/dL)	36	40	44	36
C4 (mg/dL)	6	3	7	8
Anti-SM (IU/mL)	> 100	NR	NR	NR
Anti-Ro	+	+	-	-
Hemolytic anemia	+	+	+	+
Leucopenia/lymphopenia	+	+	+	+
High CRP	+	+	+	+
Proteinuria (g/24 h)	1.8 to 21	3 to 10.76	10	ESCKD
Blood culture	*E. coli*	*E. coli*	-	-
Urine culture	*E. coli*/PA	*E. coli*	*E. coli*	*E. coli*
Treatment				
Methylprednisolone pulse	+	+	+	NU
Ⅳ IgG	+	+	NU	NU
Cyclophosphamide	NU	NU	+	NU
Oral prednisolone	+	+	+	+
Prokinetics	+	+	+	+
Antibiotics	+	+	+	+
Others				
Total parenteral nutrition	+	+	+	-
Surgery prior to diagnosis	+	+	+	+

+: Positive; -: Negative; SLE: Systemic lupus erythematosus; NU: Not used; NR: Not reported/not realized; ESCKD: End-stage chronic kidney disease; CRP: C-reactive protein; ACR: American College of Rheumatology; CT: Computed tomography; ANA: Antinuclear antibodies (normal, nondetectable); anti-dsSNA: Double-stranded DNA (positives: > 25 IU/mL); C3: C3 Complement (90-189 mg/dL); C4: C4 Complement (10-40 mg/dL); LDH: Lactate dehydrogenase; *E. coli*: *Escherichia coli*; PA: Pseudomonas aeruginosa; anti-SM: Anti-smith antibodies; Ro: Anti-Ro antibodies; SLEDAI: Systemic lupus erythematosus disease activity index; Ⅳ IgG: Intravenous human immunoglobulin; IU: International units.

lies in that a large percentage of these patients can initially present the condition together with activity at the renal, hematological and neurological levels[7,11-18]. The most frequently described treatment is based on corticosteroids or immunosuppressants. We describe four cases of intestinal pseudo-obstruction in patients with SLE associated with severe infections, with an emphasis on the fact that diagnostic delay modifies the course of the disease and may cause the patient to be subjected to surgical procedures that are not definitive and complicate the evolution.

CASE REPORT

Case 1

We present the case of a 27-year-old female. During the previous 8 mo the patient had intermittent episodes of abdominal pain and oral intolerance with a 10-kg weight loss. She was operated on during two occasions due to acute abdomen, without revealing any abnormalities. Prior to admission she had dysphagia to solid foods, dysuria and urinary incontinence, malar erythema, edema of the lower extremities and pleural effusion. Laboratory tests showed hyperazotemia, proteinuria (up to 21 g/24 h), hemolytic anemia [Hb 8 g/dL, lactate dehydrogenase (LDH) 785 mg/dL, indirect bilirubin 0.83 mg/dL, schistocytes in blood peripheral smear, positive direct Coombs], low complement, positive antinuclear antibodies, anti-DNA, anti-SM and anti-Ro (Table 1). Therefore, a diagnosis of SLE was concluded. According to imaging studies, the patient was diagnosed with intestinal pseudo-obstruction with dilatation of the pyelocalyceal systems, ureterohydronephrosis and emphysematous pyelonephritis (Figure 1). Panendoscopy was performed with report of esophagitis with esophageal hypomotility. Colovesical fistula was ruled out by colonoscopy and urethrocystography. The patient developed amaurosis fugax and seizures classified as central nervous system activity after resonance angiography, lumbar puncture and cerebral perfusion scan. *E. coli* was isolated from the urine culture with > 100000 colony-forming units (CFU). The patient developed sepsis (positive blood cultures for *E. coli*) and was given imipenem, amikacin and levofloxacin for a total of 35 d. Abdominal pain persisted along with radiographic manifestations of intestinal pseudo-obstruction. After ruling out other causes, it was decided that the clinical picture of pseudo-obstruction was secondary to SLE. Steroids were initiated for 5 d (methylprednisolone 1000 mg/d) and then oral prednisone with a reduction scheme, prokinetics (erythromycin) and total parenteral nutrition were administered. There was partial improvement in the clinical picture but without any resolution. After this first regimen, the SLE disease activity index (SLEDAI) was calculated to be 24 points. There was persistence of growth in the cultures. It was decided to administer human immunoglobulin Ⅳ at a dose of 2 g/kg weight divided into 5 d (400 mg/kg per day), after which the complement levels increased, inflammatory markers and proteinuria decreased, hemoglobin increased, and microorganisms were eradicated. Intestinal motility was finally increased, with total resolution of the GI disorder. The patient was discharged from the hospital and continued with outpatient management with mycophenolate mofetil and oral prednisone without presenting new events of GI relapse to date.

Case 2

We present the case of a 23-year-old female with a history of left optical neuritis associated with generalized erythema for 3 years. It was treated as multiple sclerosis

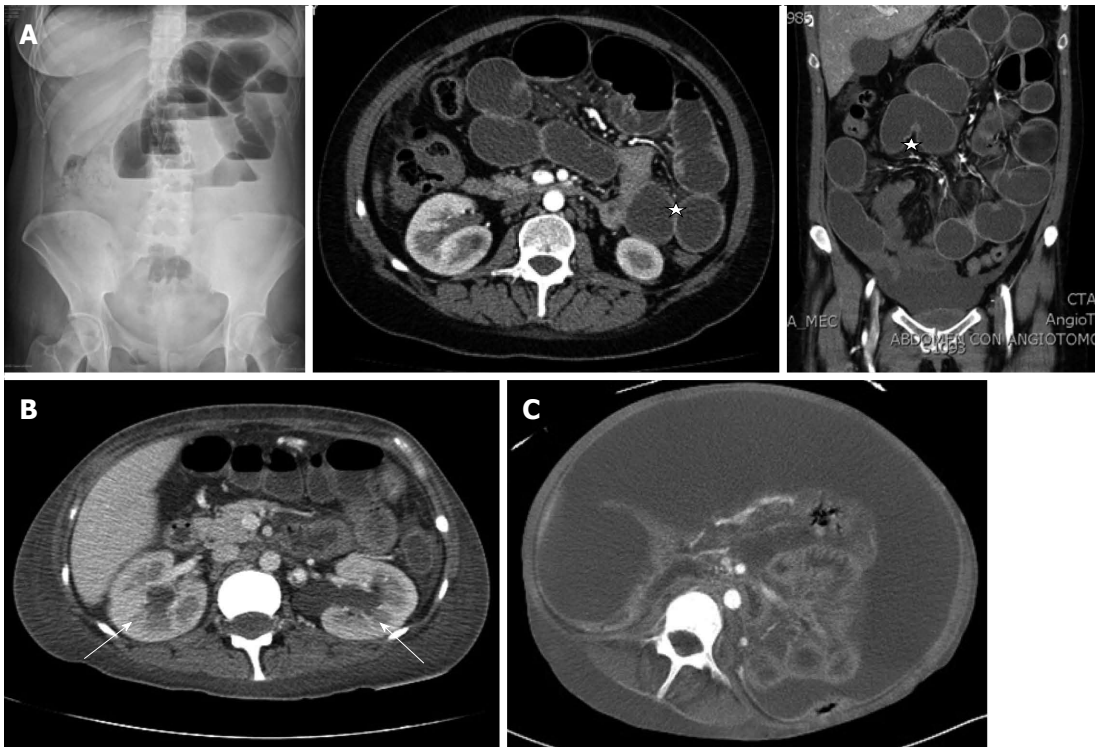

Figure 1 Imaging studies of intestinal pseudo-obstruction in lupus. A: Represents the images where signs such as distention of the small bowel loops and air fluid levels are visible and edema of the wall labeled with stars known as target lesion; B: Shows the ureterohydronephrosis with labeled arrows (in this case bilateral), which is an accompanying frequent finding; C: Represents the image of intestinal visceromegaly.

and urticarial vasculitis and managed with β-interferon and topical steroids. The patient was admitted to the hospital with generalized abdominal pain, vomiting and oral intake intolerance. She was diagnosed with acute abdomen and received two surgical interventions with findings of only ascites and splenomegaly. Three months afterwards she was readmitted with similar complaints, as well as findings of dysphagia, dysuria and a weight loss of 15 kg. ANA and anti-DNA titers were obtained with positive results, and she also had leucopenia, lymphopenia, proteinuria (up to 10.76 g/24 h), hemolytic anemia (8 g/dL, LDH 840 mg/dL, direct Coombs positive 1:6, schistocytes in FSP) and low complement (Table 1), and urinary tract infection with urine culture isolating 100000 CFU of *E. coli*. An abdominal X-ray scan reported images characteristic of intestinal obstruction, ruling out a mechanical cause (Figure 1). The patient subsequently underwent panendoscopy with manometry with reports of incompetence of the gastroesophageal junction and esophageal and duodenal hypomotility. Metabolic causes for ileus were ruled out. The patient continued with the same symptomatology with the suspected diagnosis of SLE with GI involvement. She was managed with methylprednisolone boluses at 1000 mg/d for 3 d and broad spectrum antibiotics, obtaining partial improvement of the GI symptoms. However, she developed severe sepsis (blood cultures positive for *E. coli*). Based on information of activity (SLEDAI 11) and sepsis it was decided to administer human immunoglobulin IV at a dose of 2 g/kg per total dose (400 mg/kg per day), after which the cell counts normalized, proteinuria, ANA and anti-DNAs decreased, cultures became negative and there was improvement in intestinal transit. The patient was discharged from the service, continuing on oral steroids and monthly cyclophosphamide without severe GI symptoms.

Case 3

We present the case of a 25-year-old female with a history of photosensitive malar erythema and intermittent symmetrical polyarthritis. Diarrhea, abdominal pain and vomiting had a 1-mo evolution and the patient was being managed conservatively. One week after discharge, the patient again presented with abdominal pain, vomiting, dysphagia to solids, diarrhea and fever. She arrived at the hospital where she was admitted and surgically intervened due to suspicion of acute appendicitis with insidious progression. During hospitalization she developed hypertension, edema of the lower extremities, acute renal failure and radiological picture of intestinal pseudo-obstruction (Figure 1). Hypokalemia was documented (2.9 mmol/L) and corrected. There were hemolytic anemia (direct Coombs positive 1:8, elevated LDH 479 mg/dL, schistocytes in FSP), leukopenia with lymphopenia, elevated anti-dsDNA, ANA antibodies, low complement, and proteinuria (10 g/24 h) as well as *E. coli* infection in the urine culture (> 100000 CFU). With the criteria mentioned, a diagnosis of active SLE was made (SLEDAI 18). Management with parenteral hydration was begun along with replacement of electrolytes and broad spectrum antibiotics, without improvement being noted. She

developed anuria and acute pulmonary edema and temporary hemodialysis was begun. There was no improvement in the GI symptoms, with persistent air fluid levels with thickening of the intestinal wall and ureterohydronephrosis seen on X-ray and tomographic images (Figure 1). With the infection resolved and with the possibility of severe renal damage, methylprednisolone was administered at a dose of 1000 mg for 3 d, with reduction scheme with oral prednisone and cyclophosphamide at a dose of 1 g/m^2 SC, after which renal and gastrointestinal function improved. The patient was discharged from the hospital and was managed with a monthly dose of cyclophosphamide. Abdominal symptoms recurred 2 mo later, and the patient was managed in a similar manner.

Case 4

We present the case of a 37-year-old female with a history of SLE diagnosed for 12 years. The patient was treated with prednisone, cyclophosphamide and azathioprine, progressing towards end-stage renal disease managed with peritoneal dialysis and subsequently with hemodialysis. During the 3 years prior to admission she presented recurrent pictures of diarrheal syndromes, requiring hospital management. A month prior to admission she presented the same gastrointestinal symptoms as well as fever, paresthesia, and Raynaud's phenomenon. Tomography at the time of hospitalization showed intestinal visceromegaly and bilateral uretero-hydronephrosis (Figure 1). *E. coli* was isolated from the urine culture (> 100000 CFU). Antimicrobial therapy was administered, and bacterial culture was negative after 7 d of treatment. Despite treatment, she persisted with fever, pain and abdominal distention without an obvious focus of sepsis. She developed bilateral pleural effusion, polyarthritis, leukopenia with lymphopenia, hypocomplementemia, and hemolytic anemia (positive Coombs 1:6, LDH 780 mg/dL, FSP with schistocytes). The remainder of the serological exams was not done because of the low suspicion of activity (SLEDAI 6). She was treated conservatively for 28 d with laboratory tests being within normal limits. The SLEDAI was once again calculated with a score of 9. The dose of oral steroids was increased, maintaining prednisone at 1 mg/kg weight. GI symptoms improved after 5 d. Levels of activity increased and feeding was reinitiated. Due to improvement, the patient was discharged to home. Unfortunately, she again experienced GI symptoms, occasionally not being related to data of activity in other organs. The patient was managed with steroids on readmission with partial improvement. She was lost to follow-up.

DISCUSSION

GI symptoms are rarely reported as manifestations of SLE and are rarely used as data that translate to severity[19,20]. This may be because, on one hand, they are not assumed to be part of the diagnostic criteria of SLE[3,5,9]. On the other hand, a high level of suspicion is required for making the diagnosis[11]. Because this disease has an inflammatory basis, it could affect any organ[1], which includes the GI tract and other related organs (the pancreas, liver, gallbladder, and pancreatic and biliary ducts)[4,5,8,10]. Intestinal pseudo-obstruction may be the initial manifestation in association with other organs, presenting in this manner in up to 50% of the cases[10-18] and representing a diagnostic challenge for the clinician as well as for the surgeon[13]. It is a serious disease that may compromise the life of the patient when not detected in a timely manner[5]. Patients may present symptoms of recurrent abdominal pain associated with bloating, nausea, vomiting and intolerance to oral feeding, noting that symptoms may precede the diagnosis of lupus from 11 to 66 d and even up to 2 years[5,11]. There are approximately 32 cases published in the English literature[10-18] of patients who have presented intestinal pseudo-obstruction secondary to SLE, the majority being female and of Asian origin. Medina *et al*[21] published a series of cases of acute abdomen in Mexican patients with lupus; however, emphasis was principally placed on the surgical findings where the suspicion was intestinal vasculitis and acute abdomen. The majority of the cases reported in the literature are young females, half of whom had a prior diagnosis of SLE[11-17] and who presented with a subacute progression of the GI symptoms associated, for the most part, with renal, hematological and generalized symptoms. In agreement with this information, our small sample shows the same described pattern (Table 1); 75% of the patients did not have a prior diagnosis of SLE but had renal and hematological alterations, ureterohydronephrosis, and serositis associated with an intestinal picture. They were subjected to laparotomy during more than one occasion because of suspected surgical abdomen pathology, without revealing the cause of the symptoms. In our case 4, the patient had a prior diagnosis of SLE and had a poor evolution, developing end-stage chronic renal disease requiring hemodialysis. Her history was notable for recurrent bouts of diarrhea and abdominal pain for 3 years, which would suggest a chronic course of the disease. It was not previously suspected that the symptoms may have been related with her baseline disease; therefore, directed treatment was not tested. During her latest hospitalization, steroid doses were increased with the goal of decreasing other signs and partially improved intestinal transit and feeding tolerance. Despite this, the patient continued with similar symptoms during her subsequent hospital admissions. This progression agrees with cases described by other authors, opening the possibility that the recurrence - and not the detection or early treatment - could condition a chronic type characterized by poor response to treatment and generalized dysfunction of the intestinal motility. These cases are described as generalized megaviscera[22]. It is believed that chronic sustained inflammation may trigger smooth muscle fibrosis as shown in previous histopathological reports or even in autopsies[13]. On the other hand, 100% of our patients manifested involvement of another organ or system and

were associated with positive serological markers with hypocomplementemia. Only two cases had a panendoscopy with manometry performed, which showed esophagitis and esophageal and gastric hypomotility. Only in the first case presenting with emphysematous pyelonephritis, colonoscopy and intestinal transit with barium enema were performed to rule out colovesical fistula (suspected due to emphysomatosus pyelonephritis). In terms of the rates of disease activity, 75% showed indices of Systemic Lupus International Collaborating Clinics (SLICC) and SLEDAI suggestive of activity. A notable difference found in this case series is that dysuria correlated with urinary tract infections documented by urine cultures (> 100000 CFU *E. coli* in the four patients and *Pseudomonas aeruginosa* in one patient) in addition to the isolation of the same microorganism in blood cultures (*E. coli*) in two cases.

Pathogenesis

Little is known about the pathogenesis of the disease. Etiologies such as the production of antibodies, smooth muscle myopathy, neurological involvement of the myenteric plexus and the autonomic nervous system, intestinal vasculitis with secondary ischemia, and immunocomplex deposits have been explored[10,13,14] as well as irritation of the intestine due to the presence of ascitic fluid[12] as probable mechanisms producing the disease. Intestinal sections most frequently affected are the jejunum or ileum and a high percentage of cases are associated with esophageal hypomotility and ureterohydronephrosis[5]. Some authors previously demonstrated smooth muscle motility disorders in phase Ⅲ of the migratory motor complex[13]. This theory may be supported by the damage to the muscle layer caused by the immunocomplex deposit and secondary inflammation. Production of an antibody against the smooth muscle has been proposed[4] that could cause tissue destruction, although until now it has not been demonstrated[23]. Immunocomplexes that include anti-DNA antibodies and antibodies against the collagen-like region C1q facilitate their accumulation in the glomerulus. This affinity may be shared with any tissue that expresses the appropriate receptors[1], opening the possibility of the same mechanism of injury in the intestine. The hematological disorder manifested by cytopenia is associated with the presence of anticellular antibodies $TCD3^+$/TCR and anti-Ro antibodies[4,5]; the latter have the capacity of altering the function of the myocytes and of the cell conduction systems, presumed to be another mechanism of injury[1]. For this reason, the association between leucopenia and intestinal pseudo-obstruction may not be coincidental, with it being a marker of suspicion. On the other hand, at the renal level, production of γ-interferon by the mesangium induces the production of anti-α actinin antibodies[1]. This is one of the proteins that comprise the contractile apparatus of the visceral smooth muscle, which may be a factor explaining the association between renal damage and intestinal pseudo-obstruction. Finally, some authors believe that the intestinal pseudo-obstruction may be a late manifestation of intestinal vasculitis due to the histopathological findings[4,5,24]. These may evolve to atrophy and fibrosis which, in its chronic form, would correlate with the pictures described as generalized visceromegaly[22].

Diagnosis

The clinical picture, age and gender of the patient, together with imaging and laboratory studies, constitute the first tool for the suspected diagnosis. Plain X-ray of the abdomen may provide useful information when we are dealing with an obstructive process[6] (air fluid levels, coffee ground loops, absence of air in the pelvic cavity, image of "stack of coins"); however, the noninvasive imaging study with the greatest diagnostic usefulness is abdominal computed tomography because it helps to rule out mechanical causes of the obstruction and identifies characteristic images such as target dilated loops of the jejunum and ileum, swelling of the intestinal walls, air fluid levels, pyelocaliectasis and, above all, ureterohydronephosis that may be present in up to 60% of cases[4,5,10-18]. Panendoscopy and manometry have demonstrated esophagitis and disorders of esophageal motility[13] as well as gastric and duodenal hypomotility[4,5,10,13]. Invasive methods such as colonoscopy and double balloon enteroscopy provide information on portions of the small and large intestines that cannot be reached by other means, as well as the possible advantage of taking biopsies[15]. However, it is not a method that is frequently used, given that there is currently no pattern or specific marker in the histopathological study in existence considered to be the gold standard[4,5,10]. In addition, there are reports about the predisposition of causing greater ischemia by dilation of the loops and risk of intestinal perforation and secondary peritonitis. Disease activity indexes such as SLEDAI and SLICC do not always directly correlate with gastrointestinal pictures[25]; therefore, it is difficult to consider them as useful markers. Differential diagnosis includes any disorder that causes dysmotility and is associated with extragastrointestinal symptoms. Among the most important of these are sporadic myopathies, systemic sclerosis, dermatopolymyositis, lymphoid and amyloid infiltration, to mention a few[5]. We emphasize that early detection correlates with a better prognosis and avoids unnecessary surgical procedures that hinder evolution and lead to a poor prognosis. A last resort would be exploratory laparotomy, although this should be done when there are eminent signs of intestinal perforation (free air in the abdominal cavity or septic shock in the presence of acute abdomen).

How to suspect it?

Although GI symptoms are common to the majority of the presentations, there are key data that may represent the difference in recognition of the disorder. In young patients, especially females with recurrent clinical-radiological manifestations of intestinal occlusion, an exhaustive interview should be carried out in search of manifestations or information that may be related to SLE. The

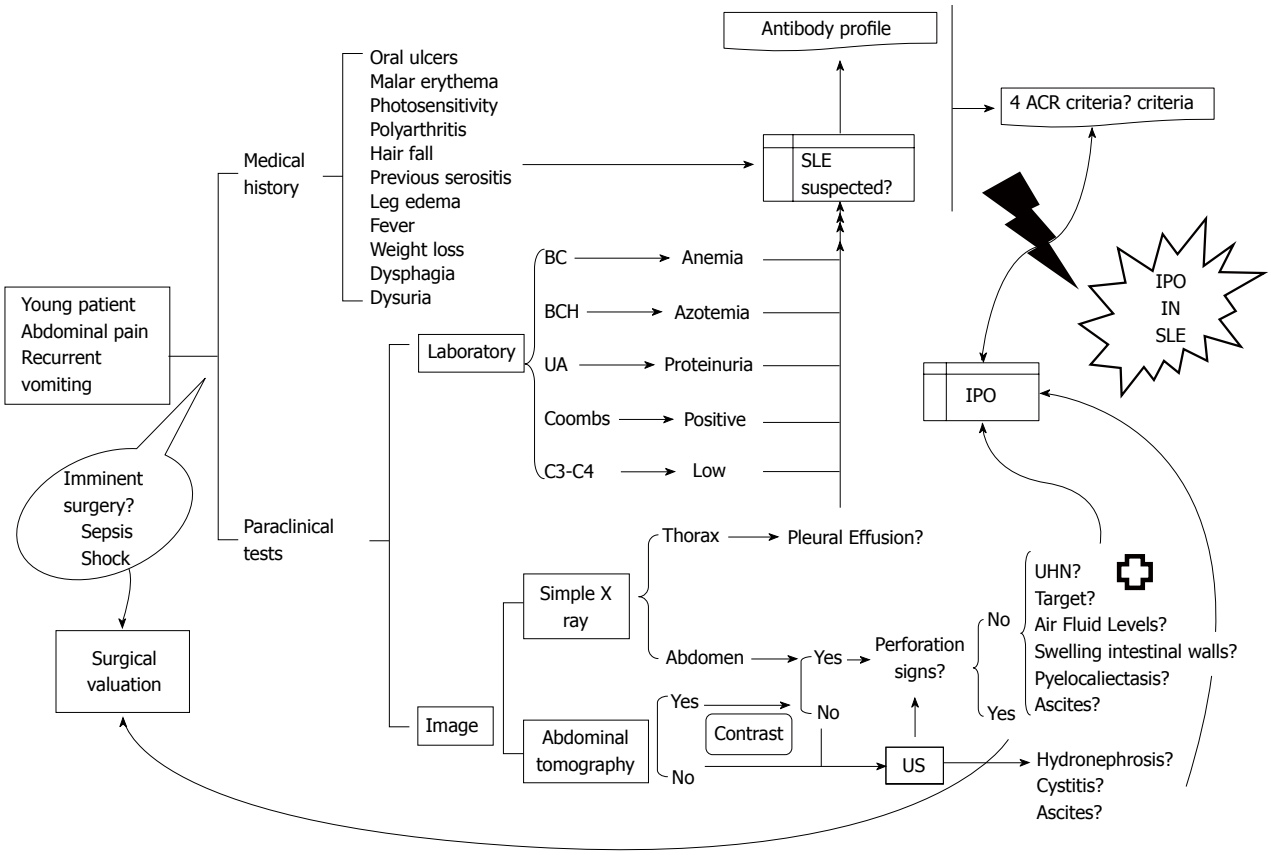

Figure 2 Suggested approach of intestinal pseudo-obstruction in lupus. When a young patient presents with a clinical-radiological picture of intestinal pseudo-obstruction, the need for urgent surgery must be ruled out at first instance. Extensive interrogation and thorough examination must be carried out along with information that could suggest the picture. Among the initial examinations are X-ray, contrast abdominal tomography if possible or renal ultrasound if not possible, along with blood and urine tests. If information is compatible with the disease, specific serology should be carried out to determine if disease criteria have been met. If the diagnosis is corroborated, the patient should be evaluated by a specialist in order to initiate timely treatment. AA: Acute abdomen; BC: Blood cytometry; BCH: Blood chemistry; UA: Urinalysis; C3-C4: Complement; US: Ultrasound; IPO: Intestinal pseudo-obstruction; SLE: Systemic lupus eythematosus; ACR: American College of Rheumatology; UHN: Ureterohydronephrosis.

initial approach should include blood cytometry, general urine examination, Coombs test and complement as firsthand resources as well as plain X-rays and contrast abdominal tomography whenever possible. If leuko/lymphopenia, anemia with positive Coombs reaction, proteinuria, low complement and ureterohydronephrosis are found, a diagnosis of intestinal pseudo-obstruction in SLE should be suspected and confirmed (Figure 2).

Treatment

Treatment should be individualized and generally multidisciplinary[1]. Among the recommendations for management of SLE issued by the European League Against Rheumatism and established by the ACR, there is no scheme suggested for GI manifestations[3,9]. The most frequently described experience in disease management is based on corticosteroids administered in pulses (methylprednisolone 500-1000 mg daily for 2-5 d) associated with other immunosuppressants (cyclophosphamide being the most used)[10-18]. Cases have been reported where cyclosporine A, methotrexate, azathioprine[7,11,24], and tacrolimus were used[26]. The use of prokinetics should be considered, preferably erythromycin because of its antimicrobial properties, although the use of metoclopramide, octreotide and neostigmine should be considered. Finally, use of parenteral nutrition should be individualized in cases where oral feeding is not possible. Human immunoglobulin is extracted from the plasma of thousands of donors[27,28] and is mainly composed of a pool of antibodies, mainly IgG (97%). Its use has been described in reports of cases and small cohorts with good results[2,29-31]. The usual administered dose is 2 g/kg divided into 2-5 d in continuous IV infusion. The described mechanisms of action vary[27,29,32-35]. It is considered more than an immunosuppressive treatment and immune regulatory agent that obviates the toxic and teratogenic effects of other drugs, providing a favorable safety profile for infected patients[29,35]. Any prior condition should be taken into consideration, which may contraindicate its use along with the problem of its high cost, which hinders its accessibility for treatment[27]. Early directed treatment makes progression of the disorder highly reversible, avoiding the need for surgery and having an impact on survival, recovery of function and decrease in complication rates[4,19,22,25].

Intestinal pseudo-obstruction secondary to lupus is an infrequent disorder that may initially manifest together with other organ and system involvement. It should be suspected, especially in young women with clinical-radio-

logical manifestations and information of disease activity at another level. Urinary tract infections may be associated with urinary manifestations and complicate symptom progression. Management of the disorder should include immunomodulators, principally corticosteroids and cyclophosphamide, prokinetics, and parenteral nutrition when required. In our short experience we believe that use of IV human immunoglobulin may be a good alternative where there are concomitant severe infection or sepsis and disease activity if there is no contraindication for its use.

ACKNOWLEDGMENTS

We thank Sharon Morey for technical and editorial assistance.

COMMENTS

Case characteristics

Intestinal pseudo-obstruction may be the initial manifestation in association with other organs, presenting symptoms of recurrent abdominal pain associated with bloating, nausea, vomiting and intolerance to oral feeding.

Clinical diagnosis

The clinical picture, together with imaging and laboratory studies, constitutes the first tool for the suspected diagnosis; however, the study with the greatest diagnostic usefulness is abdominal computed tomography.

Differential diagnosis

Differential diagnosis includes any disorder that causes dysmotility and is associated with extra-gastrointestinal symptoms (sporadic myopathies, systemic sclerosis, dermatopolymyositis, lymphoid and amyloid infiltration).

Laboratory diagnosis

There are no characteristic findings of the disease. Leuko/lymphopenia, anemia with positive Coombs reaction, azotemia, proteinuria/erythrocyturia and low complement levels are the most frequent.

Imaging diagnosis

Abdominal computed tomography rules out mechanical causes and identifies characteristic images. Panendoscopy and manometry have demonstrated esophagitis and disorders of esophageal, gastric and duodenal motility.

Pathological diagnosis

There is currently no pattern or specific marker in the histopathological study in existence considered to be the gold standard. Damage to the muscle layer caused by the immunocomplex deposit and secondary inflammation with intestinal vasculitis are some frequent findings.

Treatment

The most frequently described experience in disease management is based on corticosteroids administered in pulses associated with other immunosuppressants (cyclophosphamide being the most used). Prokinetics should be considered and use of parenteral nutrition should be individualized in cases where oral feeding is not possible.

Experience and lessons

Early recognition and directed treatment make progression of the disorder highly reversible, avoiding the need for surgery and having an impact on recovery of function and decrease in complication rates. We believe the use of IV human immunoglobulin with steroids may be an alternative only where there are complicated infections or sepsis and disease activity.

Peer review

According to reviewers, the present report is new according to the point that it shows that many cases present acute abdomen previous to the diagnosis of SLE, and physicians should be aware of intestinal pseudo-obstruction due to SLE when they encounter a patient with acute abdomen with a doubtful cause.

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P- Reviewer: Tomita Y **S- Editor**: Qi Y **L- Editor**: Wang TQ **E- Editor**: Wang CH

World Journal of Gastroenterology

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DOI: 10.3748/wjg.v20.i32.11451

World J Gastroenterol 2014 August 28; 20(32): 11451-11455
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Three-dimensional imaging identified the accessory bile duct in a patient with cholangiocarcinoma

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Received: January 10, 2014 Revised: March 11, 2014
Accepted: April 21, 2014
Published online: August 28, 2014

Abstract

The development of diagnostic imaging technology, such as multidetector computed tomography (MDCT) and magnetic resonance cholangiopancreatography (MRCP), has made it possible to obtain detailed images of the bile duct. Recent reports have indicated that a 3-dimensional (3D) reconstructed imaging system would be useful for understanding the liver anatomy before surgery. We have investigated a novel method that fuses MDCT and MRCP images. This novel system easily made it possible to detect the anatomical relationship between the vessels and bile duct in the portal hepatis. In this report, we describe a very rare case of extrahepatic cholangiocarcinoma associated with an accessory bile duct from the caudate lobe connecting with the intrapancreatic bile duct. We were unable to preoperatively detect this accessory bile duct using MDCT and MRCP. However, prior to the second operation, we were able to clearly visualise the injured accessory bile duct using our novel 3D imaging modality. In this report, we suggest that this imaging technique can be considered a novel and useful modality for understanding the anatomy of the portal hepatis, including the hilar bile duct.

Key words: 3-dimensional imaging; Hepatobiliary and pancreatic surgery; Accessory bile duct; Caudate lobe bile duct; Cholangiocarcinoma

Core tip: We present a case study in which 3-dimensional (3D) images were reconstructed to detect a case of extrahepatic cholangiocarcinoma associated with an accessory bile duct from the caudate lobe connecting with the intrapancreatic bile duct. We could not detect this condition preoperatively using standard imaging techniques; however, the 3D reconstruction enabled us to visualise the bile duct and treat the patient successfully.

Miyamoto R, Oshiro Y, Hashimoto S, Kohno K, Fukunaga K, Oda T, Ohkohchi N. Three-dimensional imaging identified the accessory bile duct in a patient with cholangiocarcinoma. *World J Gastroenterol* 2014; 20(32): 11451-11455 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11451.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11451

INTRODUCTION

In hepatobiliary and pancreatic surgery, it is important to avoid injury by taking note of any anomalies of the extrahepatic bile duct. According to previous reports, the accessory bile duct is of particular importance because it occurs relatively frequently[1-5]. It is well known that there is significant variation in the caudate lobe bile duct[6-8]. Recently, Ryu[8] were able to visualise the variation in the caudate lobe bile duct using 3-dimensional (3D) imaging

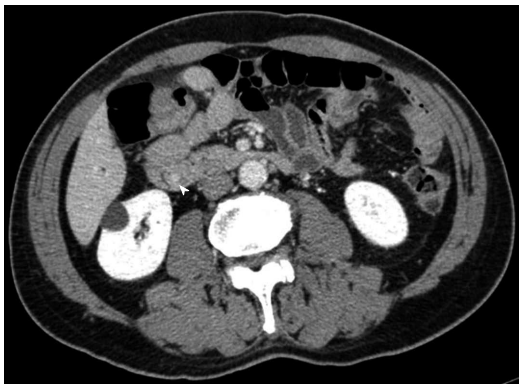

Figure 1 Abdominal computed tomography angiography image of the common bile duct. Thickening of the wall of the distal common bile duct was observed (arrowhead).

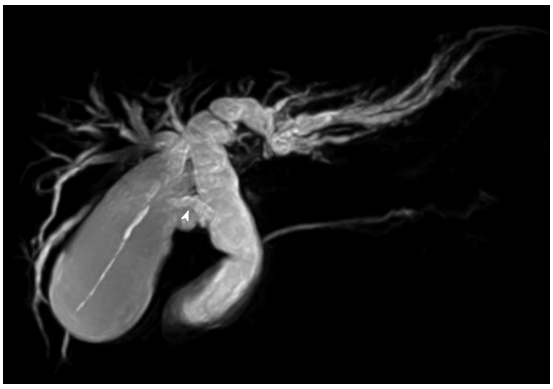

Figure 2 Magnetic resonance cholangiopancreatography. The common and intrahepatic bile ducts were dilated. It was impossible to detect the accessory bile duct preoperatively (arrowhead).

techniques.

Recent advances in diagnostic imaging technology, such as endoscopic retrograde cholangiopancreatography (ERCP) and magnetic resonance cholangiopancreatography (MRCP), have enabled us to obtain detailed information of the bile duct before surgery. However, when the common bile duct is dilated, a thin ectopic bile duct may be hidden behind it[9,10]. Moreover, with the use of MRCP alone, it is impossible to know the relative positions of the bile duct with vascular components, such as the hepatic artery and portal vein, and the parenchymal organs, such as the pancreas and liver.

Many institutes in Japan have recently begun to construct 3D images from multidetector computed tomography (MDCT) datasets for patients who undergo hepatic resection in order to share the images with the surgical staff[11-14]. We have reconstructed 3D images of the liver using the Synapse Vincent medical imaging system (Fujifilm Medical, Tokyo). Furthermore, we have developed a novel 3D imaging system by integrating MDCT and MRCP images and have applied this 3D imaging technique to hepatobiliary and pancreatic surgery[15].

In this report, we treated a very rare case of extrahepatic cholangiocarcinoma associated with an accessory bile duct from the caudate lobe connected to the intrapancreatic bile duct. The first preoperative imaging studies did not reveal the accessory bile duct, but the 3D studies conducted before the second operation visualised the duct and directed us to the source of the bile leak, a finding that greatly facilitated the second operation. We suggest that preoperative 3D imaging of the bile duct can provide useful guidance during surgery.

CASE REPORT

The patient was a 65-year-old male who was referred to our hospital because of jaundice. The patient was diagnosed with extrahepatic cholangiocarcinoma (cT1N0M0, Stage I) according to the Union Internationale Contre le Cancer (UICC) guidelines on the basis of a careful imaging study, including MDCT (Figure 1), MRCP (Figure 2) and ERCP. Neither our group nor the radiologists were able to preoperatively detect an accessory bile duct from the caudate lobe. The patient underwent a subtotal stomach-preserving pancreaticoduodenectomy. Because of refractory bile leakage 5 d after the operation, tubography was undertaken through the biliary drainage tube and the abdominal drainage tube. In this examination, leakage of the contrast media from the end-to-side biliojejunostomy was not observed. However, leakage was evident in images of the injured site of the bile duct (Figure 3). Furthermore, in order to clarify the injury site of the bile duct, fusion-3D images were created from preoperative MDCT and MRCP images (Figure 4). This 3D image enabled us to detect the injured accessory bile duct from the caudate lobe. Nineteen days after the first operation, the patient underwent a second operation. The intraoperative findings showed the orifice of the accessory bile duct on the surface of the liver that had been preoperatively identified on inspection of the 3D images (Figure 5). We confirmed that the bile leak was from the accessory bile duct. Abdominal drainage and an end-to-side biliojejunostomy for the injured accessory bile duct were performed. The patient had a favourable postoperative course and was discharged 16 d after the second operation. According to the UICC guidelines, the pathological classification of the tumour was pT2N1M0, Stage ⅡB. At the present time, the patient is currently alive without recurrence 240 d after the first operation.

DISCUSSION

Healey defined the accessory bile duct as an extrahepatic bile duct, without a bile duct connecting with the common hepatic duct within a liver[1]. Miyakawa[3] reported an accessory bile duct in 21 out of 450 (4.7%) patients. Similarly, Hisatsugu[2] reported an accessory bile duct in 616 out of 19892 (3.1%) patients undergoing bile duct surgery. They classified the accessory bile duct into seven categories based on the position on the common bile duct. In the present case, the accessory bile duct connected to the inferior common bile duct and was classified as

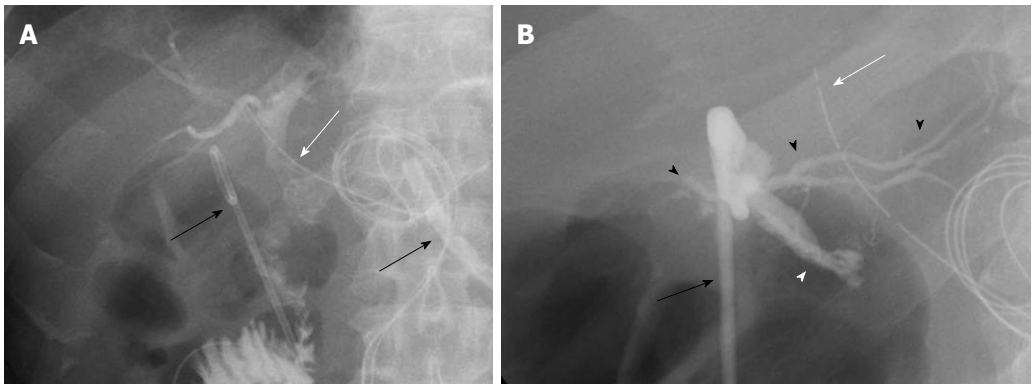

Figure 3 Tubography through the biliary drainage tube (white arrow) and the abdominal drainage tube (black arrow) after the first operation. A: The radiological image indicated the intrahepatic bile duct, common bile duct, and jejunum. Leakage of the contrast media from the end to side biliojejunostomy was not observed. Two abdominal drainage tubes (black arrows) were inserted into the abdominal cavity; B: Postoperative tubography through the abdominal drainage tube (black arrow) was performed, which produced the images of the injured site of the probable caudate bile duct (white arrowhead) and the other intrahepatic bile duct (black arrowheads). The biliary drainage tube is shown (white arrow).

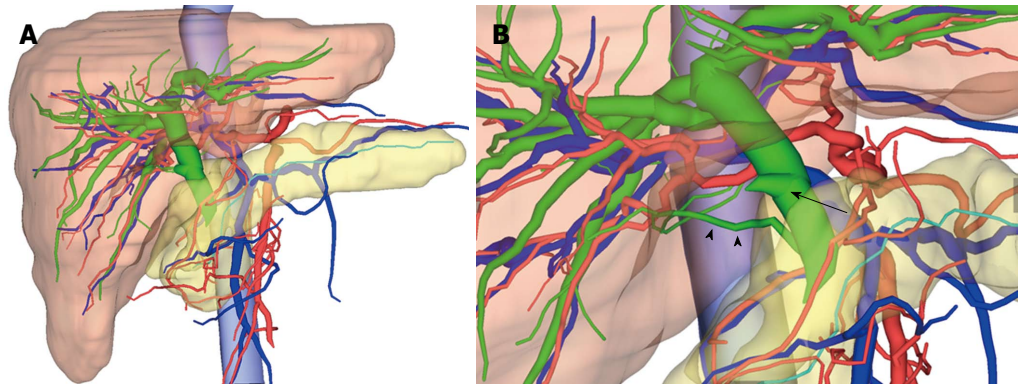

Figure 4 3-dimensional images by integrating multidetector computed tomography and magnetic resonance cholangiopancreatography images. A: 3-dimensional (3D) image view from the front side of the patient. The red colour represents the arteries, the blue represents the veins and the portal vein, the green represents the biliary duct, and the turquoise represents the pancreatic duct. We were able to observe that the common and intrahepatic bile ducts were dilated; B: From the 3D image view from the patient's right side, the accessory bile duct from the caudate lobe connecting to the intrapancreatic bile duct (arrowheads) was easily recognisable. The cystic duct (arrow) has branched from the middle bile duct. We were able to determine that the injured site was the accessory bile duct from the caudate lobe.

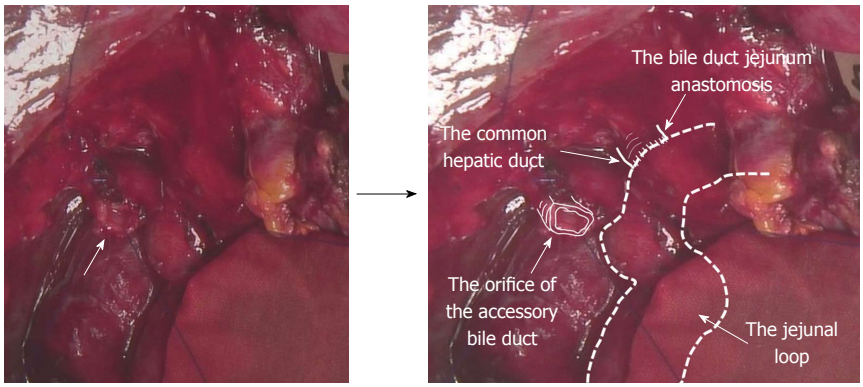

Figure 5 Intraoperative findings in the second operation. The orifice of the injured caudate lobe bile duct of the proximal side on the surface of the liver was recognised (arrow). The precise anatomy is described using the annotation method.

Type IV, which comprises 3.0% of the total.

Since the study by Kumon[6] involving the use of a flexible cast, various studies have examined the caudate lobe bile duct using techniques such as radiographic evaluation. Horiguchi *et al*[7] examined the caudate lobe bile duct in 77 patients and reported that, in approximately 70% of cases, the bile duct from the left caudate lobe connected to the left hepatic duct. Using 3D im-

Table 1 Cases of the caudate lobe bile duct connecting with the intrapancreatic bile duct

Ref.	Age	Sex	Diagnosis	Procedure
Ng *et al*[9]	1	F	Congenital dilation of the common bile duct	Extrahepatic bile duct resection
Aoki *et al*[16]	72	M	Gallbladder cancer	Extended cholecystectomy + extrahepatic bile duct resection
The present case	65	M	Extrahepatic cholangiocarcinoma	Subtotal stomach-preserving pancreaticoduodenectomy

M: Male; F: Female.

ages, Ryu[8] demonstrated that 44% of Spiegel lobe bile ducts connected to the left hepatic duct and that 90% of the caudate lobe protrusion bile ducts connected to the posterior segment bile duct. We searched Japana Centra Revuo Medicina and PubMed for reported cases of an accessory bile duct connected to the intrapancreatic bile duct and found that our case was the third to be reported[9,16] (Table 1).

Hepatobiliary and pancreatic surgery requires detailed preoperative examination of the anatomy of the bile duct in order to prevent intraoperative bile duct injury. Although it is possible to obtain detailed information preoperatively because of the advances in diagnostic imaging technology such as MRCP, there is the possibility that an anomalous thin caudate lobe branch may not be detected in patients with a dilated common hepatic duct, as in our case[3,4]. We developed 3D images by integrating MDCT and MRCP images that can produce accurate preoperative anatomical images[15]. We have applied this method for the depiction of hepatic anatomy prior to hepatobiliary and pancreatic surgery. By integrating these two images, it is possible to understand the anatomical relationships between the bile duct arrangement and the vascular components, such as the hepatic artery and portal vein, and the parenchymal organs, such as the pancreas and the liver. Moreover, it became possible to share the anatomical images with the surgical staff by sharing the 3D images preoperatively.

With regard to the therapeutic method for bile duct injury, we have to consider the diameter of the bile duct and the area it covers. Longmire *et al*[17] reported that when the bile duct has a diameter of less than 1 mm, it can potentially be ligated if there is no infection; however, a bile duct with a diameter of 2 mm or more needs to be reconstructed. Hachisuka *et al*[18] also performed intraoperative bile duct imaging and evaluated the necessity of reconstruction by examining the extent of the area covered by the bile duct. In the present case, we performed a reconstruction without hesitation because it was clear that the injured site of the bile duct was the caudate lobe branch based on the 3D images. Intraoperatively, we found that the diameter was 2 mm or more.

In conclusion, we experienced a very rare case of extrahepatic cholangiocarcinoma associated with the accessory bile duct from the caudate lobe connected to the intrapancreatic bile duct, which was revealed using 3D images. A 3D imaging system such as our new modality for preoperative assessment is considered to be useful and reliable for hepatobiliary and pancreatic surgery.

COMMENTS

Case characteristics

In this report, authors describe a very rare case of extrahepatic cholangiocarcinoma associated with an accessory bile duct from the caudate lobe connecting with the intrapancreatic bile duct.

Clinical diagnosis

They were unable to preoperatively detect this accessory bile duct using multidetector computed tomography and magnetic resonance cholangiopancreatography.

Differential diagnosis

However, prior to the second operation, authors were able to clearly visualise the injured accessory bile duct using our novel 3-dimensional (3D) imaging modality.

Imaging diagnosis

In this report, they suggest that this imaging technique can be considered a novel and useful modality for understanding the anatomy of the portal hepatis, including the hilar bile duct.

Peer review

This report is well organized and documented for the significance of 3D imaging identified the accessory bile duct in a patient with cholangiocarcinoma.

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P- Reviewer: Chang JH, Kim S, Sartelli M **S- Editor**: Gou SX
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DOI: 10.3748/wjg.v20.i32.11456

World J Gastroenterol 2014 August 28; 20(32): 11456-11459
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Rare case of omentum-wrapped abscess caused by a fish bone penetrating the terminal ileum

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Author contributions: Wu CX, Wu BQ, and Jiang Y performed the operation; Duan YF and Sun DL collected case data; Wu CX and Wu BQ wrote the manuscript; Sun DL and Jiang Y proofread and revised the manuscript; all authors approved the version to be published.

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Received: January 12, 2014 Revised: March 18, 2014
Accepted: May 12, 2014
Published online: August 28, 2014

Abstract

Accidentally ingested foreign bodies, for the most part, pass through the gastrointestinal tract, but can cause several complications. Perforation is rare, but can occur in any segment of the gastrointestinal tract. Intestinal perforations due to foreign bodies are rarely diagnosed preoperatively as clinical symptoms are non-specific and they can mimic other abdominal conditions. We describe a case of a 48-year-old patient who was admitted to the emergency room because of severe abdominal pain of 5 d duration. A computed tomography scan showed an undefined liquid collection involving a linear image 35 mm in size, suggestive of a foreign body. On laparotomy, an abscess containing a fish bone was resected. As fish bone ingestion is usually not remembered by the patient, the diagnosis can be delayed. The preoperative diagnosis is frequently acute abdomen of unknown cause. A low threshold of suspicion along with a good clinical history and radiological studies are extremely important in order to make a correct diagnosis.

Key words: Foreign body; Fish bone; Terminal ileum perforation

Core tip: Perforations due to fish bones are rare and have nonspecific symptoms, mimicking other abdominal conditions. A patient attended the emergency room due to severe abdominal pain of 5 d duration. A computed tomography scan showed an undefined liquid collection involving a linear image 35 mm in size. On laparotomy, an abscess containing a fish bone was resected.

Wu CX, Wu BQ, Duan YF, Sun DL, Jiang Y. Rare case of omentum-wrapped abscess caused by a fish bone penetrating the terminal ileum. *World J Gastroenterol* 2014; 20(32): 11456-11459 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11456.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11456

INTRODUCTION

Foreign body ingestion is frequent and can cause several complications, perforation being the most frequent[1-3]. Perforation due to ingested foreign bodies in the adult population is most common secondary to accidental ingestion and is frequently caused by dietary foreign bodies, especially fish bones. Foreign bodies usually pass through the gastrointestinal tract without problems once beyond the esophagus[3,4]. Perforation occurs in about 1% of all foreign bodies ingested usually due to long and sharp objects such as fish bones, toothpicks, chicken bones and needles[3,4]. We herein report the diagnosis and treatment of a patient with omentum-wrapped abscess caused by a

fish bone penetrating the terminal ileum.

CASE REPORT

A 48-year-old male patient attended the emergency room due to severe abdominal pain, mainly in the middle quadrants, of 5 d duration, without nausea or vomiting. Gastrointestinal transit was normal, without bleeding per rectum. There was no history of anorexia or weight loss. Respiratory and urinary symptoms were absent. On physical examination, the patient had generalized tenderness of the abdomen, which was maximal in the middle upper quadrant, signs of peritoneal inflammation with guarding, rebound, and tap tenderness, without Rovsing's or Murphy's sign, and peristaltic sounds were not audible. He was tachycardic with a fever of 39.6 ℃. White blood cells count was 11.96 × 10^9/μL, no anemia was found, and hepatic and pancreatic tests were normal.

Abdominal ultrasound showed peritoneal effusion, a normal gallbladder, and the ileo-cecal appendix was not visualized. An abdominal computed tomography (CT) scan was performed, and in addition to moderate peritoneal effusion, an undefined liquid collection involving a linear image 35 mm in size was evident in the omental abscess, suggestive of a foreign body (Figure 1A).

When asked about his diet, the patient mentioned that some days earlier he had eaten fish, however, foreign body ingestion was not remembered. On this basis, a diagnosis of bowel perforation due to a foreign body (fish bone) was made.

Systemic antibiotics were initiated, and a median laparotomy was performed, revealing a purulent peritoneal effusion and an abscess in the greater omentum adjacent to the transverse colon. A fibrotic closed fistula between the terminal ileum and the abscess was found (Figure 1C), which was sectioned, and the abscess was resected *en-bloc*. There was no evident colon defect, and we performed a purse-string suture in the ileum side of the fibrotic fistula and an omental patch was used to cover it. Peritoneal lavage was carried out using saline solution and a drain was placed in the abdominal cavity. When the abscess was opened, a fish bone of approximately 35 mm was found (Figure 1B).

No complications occurred after surgery, the drain was removed on the fourth post-operative day (drainage was always serous), and the patient was discharged free of symptoms seven days after surgery. During the follow-up period of 3 mo, no sequelae were observed.

DISCUSSION

Perforation can occur in any segment of the gastrointestinal tract[2-4], however, the most common sites of perforation are in the distal ileum[5-7], the cecum and the left colon[8] due to their great angulation[3,4]. Most perforations occur in the straits and the angles of the gastrointestinal tract[9]. Ingestion of foreign bodies, although a fairly common problem in pediatrics, is relatively rare in adults and is mainly found in individuals with psychiatric disorders such as bipolar disorder, depression, or post-traumatic stress disorder[10]. Alcoholism, psychiatric illness, age extremes and the use of dentures are risk factors for foreign body ingestion[3,4]. Intestinal perforation by fish bones is rare, however, their ingestion is common. The risk of perforation is related to the length and shape of the object ingested[11]. The average time from ingestion of the foreign body to perforation is 10.4 d[12]. Clinical presentation can vary with acute or chronic symptoms[2,3]. Bowel perforation by foreign bodies can mimic other abdominal conditions such as acute appendicitis, acute diverticulitis, and perforated peptic ulcer[13]. As the patient usually does not remember fish bone ingestion, diagnosis can be delayed, with months between ingestion and perforation[1,3,4]. Fish bones located in a narrow segment of the bowel can erode the mucosa, causing bacterial dissemination. As this pathological process continues, perforation and an extramural abscess occur, which leads to acute abdominal pain[3]. In the case presented here, the patient had eaten fish some days before and fish bone ingestion was not remembered, however, as a fibrotic closed fistula was found, the ingestion causing perforation was thought to have occurred earlier.

X-rays can be used to detect foreign bodies. Plain radiography is helpful in locating metallic foreign bodies and pneumoperitoneum. As the perforation hole is small and normally covered with fibrin and omentum, pneumoperitoneum is rare, being present in only 20% of patients[3,4]. CT scanning is the most accurate exam with fish bones appearing like linear images with calcic density inside an inflamed area[3]. However, CT scanning has some weaknesses: lack of awareness by the radiologist if there is no clinical suspicion; the use of oral and/or intravenous contrast can make it difficult to visualize fish bones[3]. Colon perforation can have the same radiologic and pathologic characteristics as intestinal inflammatory disease[14]. CT scanning was used in our patient to aid the diagnosis and showed a linear image inside a liquid collection suggestive of a foreign body, without pneumoperitoneum.

Treatment depends on the age of the patient and symptoms, the nature and type of foreign body and anatomical location, especially if impacted. The management may consist of conservative or interventional methods, endoscopic, laparoscopic or open surgery. Surgery is the treatment of choice for bowel perforation, and is most commonly performed by laparotomy due to its advantages in localizing the perforation, closure or repair of the defect, and peritoneal lavage. However, laparoscopy has been reported in some studies to be as good as laparotomy[15]. In our case, laparotomy was chosen, and the abscess adjacent to the transverse colon was resected en-bloc to avoid spreading pus into the abdominal cavity, and peritoneal lavage was performed to clean the purulent effusion. No defect was found in the terminal ileum,

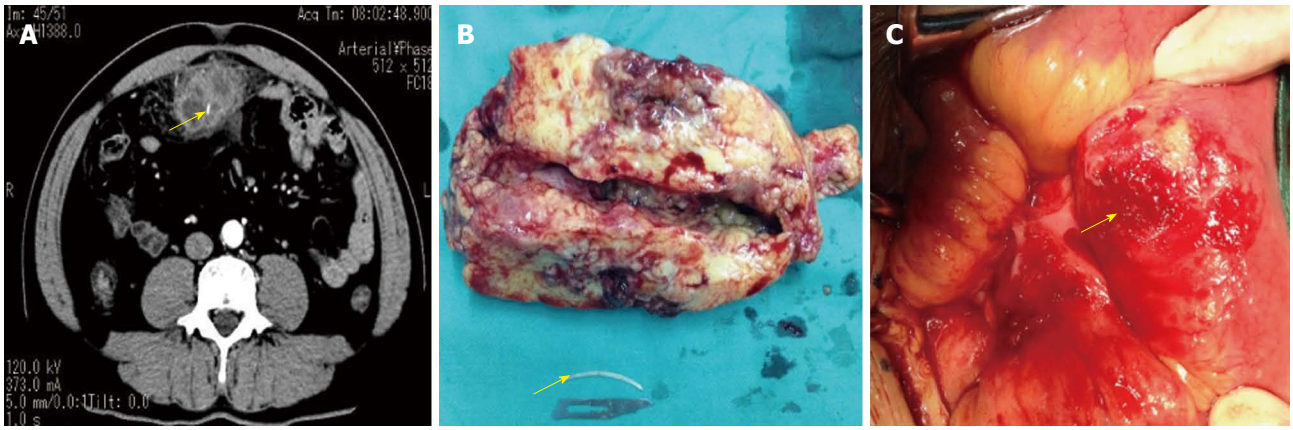

Figure 1 Computed tomography, resected abscess and fish bone and the lacation of perforation in terminal ileum. A: Axial and sagittal slides of abdominal computed tomography scan. It is visible an undefined liquid collection involving a linear image suggestive of foreign body (arrow); B: The resected abscess size and fishbone (arrow); C: The perforation in terminal ileum (arrow).

as the fistula was fibrotic and occluded, this may have been because there was a long time between perforation and surgery, with the greater omentum tapering the defect. Intestinal perforation treatment usually involves bowel resection, however, the most common treatment is suture of the perforation[8]. For this reason, the closed fistula was sectioned and a purse-string suture was performed and covered with an omental patch for safety. The patient was discharged free of symptoms on the 7th post-operative day, which is consistent with other similar case reports.

COMMENTS

Case characteristics

A 48-year-old patient attended the emergency room due to severe abdominal pain of 5 d duration.

Clinical diagnosis

On laparotomy, an abscess containing a fish bone was resected.

Differential diagnosis

Bowel perforation by foreign bodies can mimic other abdominal conditions such as acute appendicitis, acute diverticulitis, and perforated peptic ulcer.

Laboratory diagnosis

The patient was tachycardic with a fever of 39.6 ℃. Blood samples revealed a white blood cells of 11.96 × 10^9/μL, no anemia, and normal hepatic and pancreatic tests.

Imaging diagnosis

A computed tomography scan showed an undefined liquid collection involving a linear image 35 mm in size, suggestive of a foreign body.

Pathological diagnosis

Fish bone, fat and fibrous tissue with chronic suppurative inflammation, multifocal abscess formation, and histiocytosis.

Treatment

Laparotomy was performed and the abscess was resected *en-bloc* to avoid spreading pus into the abdominal cavity.

Experiences and lessons

A low threshold of suspicion along with a good clinical history and radiological studies are extremely important in order to make a correct diagnosis.

Peer review

The authors reported a patient with omentum-wrapped abscess caused by a fish bone penetrating the terminal ileum, and presented the imaging and surgical findings of the location of the terminal ileum perforation, the size of the abscess and the length of the fish bone in the abscess.

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P- Reviewer: Faenza S, Skok P **S- Editor**: Gou SX **L- Editor**: A
E- Editor: Zhang DN

World Journal of Gastroenterology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i32.11460

World J Gastroenterol 2014 August 28; 20(32): 11460-11462
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Role of over the scope clips in the management of iatrogenic gastrointestinal perforations

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Author contributions: Changela K and Virk MA contributed to study concept and design, drafting of the manuscript, technical support, study supervision; Changela K and Virk MA contributed equally; Patel N contributed to acquisition of data and images, drafting of the manuscript; Duddempudi S, Krishnaiah M and Anand S contributed to critical revision of the manuscript for important intellectual content.

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Received: January 23, 2014 Revised: March 8, 2014
Accepted: April 15, 2014
Published online: August 28, 2014

Abstract

Advances in endoscopic and surgical techniques have increased the frequency and complexity of these procedures and associated complications such as gastrointestinal perforation. With the advancements in the field of gastroenterology, the promising use of an over the scope clips (OTSC) has fulfilled the unmet need for a reliable endoscopic devise in approximation of gastrointestinal perforation. This novel approach has raised the level of confidence in endoscopist in dealing with this serious complication during endoscopy. Here we have shared our experience with OTSC to evaluate its efficacy and safety in managing iatrogenic gastrointestinal perforations during endoscopy.

Key words: Gastrointestinal perforation; Over-the-scope clip; Over the scope clip system; Endoscopic adverse events

Core tip: Gastrointestinal perforation is a frequently encountered, inevitable, and serious complication encountered during endoscopic procedures. In this editorial, we have described our experience with over the scope clip system as a promising therapeutic option in dealing with iatrogenic gastrointestinal perforations. We believe that our experience with this emerging therapeutic approach on three patients will definitely be an excellent asset to available literature and will attract more readers.

Changela K, Virk MA, Patel N, Duddempudi S, Krishnaiah M, Anand S. Role of over the scope clips in the management of iatrogenic gastrointestinal perforations. *World J Gastroenterol* 2014; 20(32): 11460-11462 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11460.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11460

TO THE EDITOR

Endoscopic adverse events are inevitable and gastrointestinal perforation is one of the serious complications encountered during endoscopic procedures. Therapeutic options are limited in fragile patients with comorbid conditions. Incidence of perforation during diagnostic endoscopy is reported to be between 0.01% and 0.6% and therapeutic endoscopy between 0.6% and 5.5%[1]. By using over the scope clip (OTSC) technique, immediate closure of these perforations would be possible during endoscopy and would increase an endoscopist's level of comfort[2]. OTSC has shown its encouraging results in management of closure of gastrointestinal fistulas, anastomotic leaks, bleeding lesions, post bariatric surgery complications and closure of gastrostomies during natural orifice transluminal endoscopic surgery[3,4]. The following three cases describe the therapeutic efficacy and technical feasibility of OTSC system in management of iatrogenic gastrointestinal tract perforations.

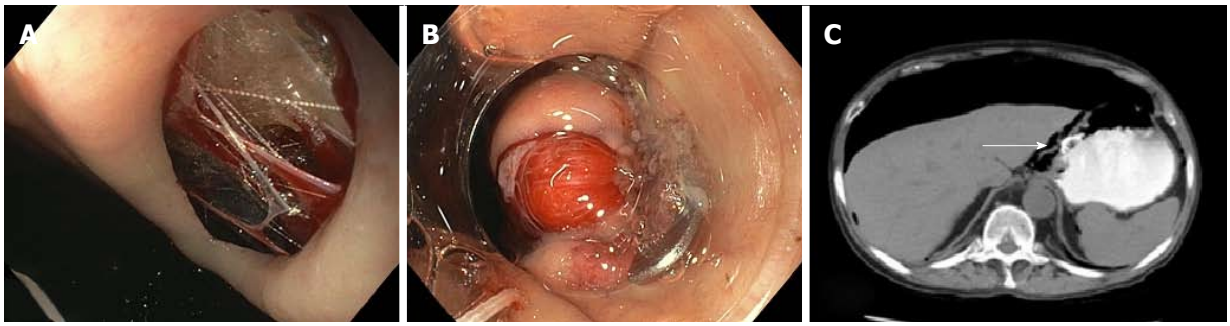

Figure 1 Endoscopic image showing linear 2 cm tear at lesser curvature (A), endoscopic image showing successful closure of the tear with an over the scope clips (B); computed tomography scan of abdomen and pelvis with contrast showing successful closer of tear with clip and no gastrografin leakage (C).

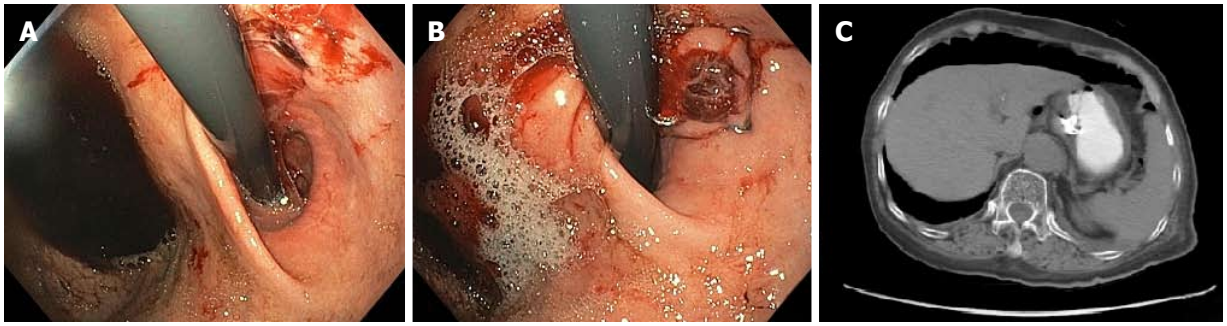

Figure 2 Endoscopic image showing a deep liner and wide tear of 1 cm with base seemed to have fibrinous appearance (A), endoscopic image of successful closure of the tear with an over the scope clips (B); computed tomography scan of abdomen and pelvis with contrast showing successful closer of tear with clip and no gastrografin leakage (C).

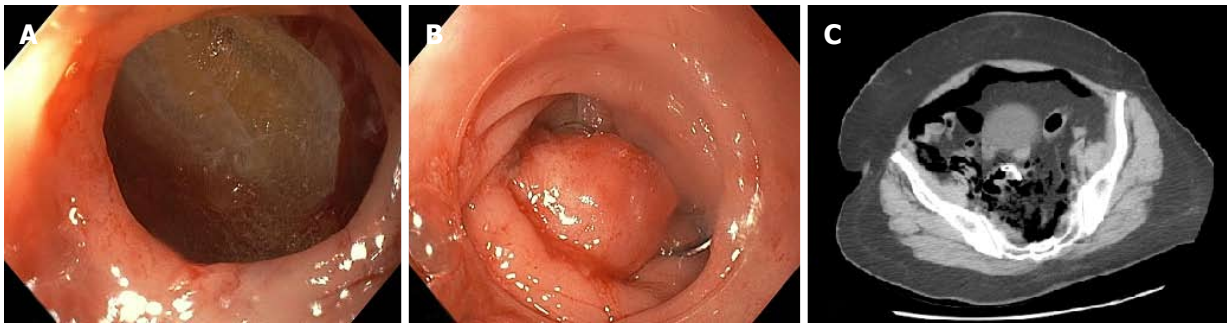

Figure 3 Endoscopic image showing area of yellow looking defect possibly omentum or serosa (contained perforation) (A), endoscopic image showing successful closure of the tear with an over the scope clips (B), computed tomography scan Image of successful closer of tear with clip without leakage of contrast (C).

In the first case, a 61 years old female with sarcoidosis underwent esophagogastroduodenoscopy for evaluation of dysphagia. Two areas of angioectasia were found in duodenum and were cauterized with a gold probe. Upon withdrawal of the scope, a linear 2 cm tear was seen in the lesser curvature about 5 cm below the gastroesophageal junction (Figure 1A). An OTSC 12/6 GC was successfully deployed endoscopically and satisfactory closure of the perforation was observed (Figure 1B). Post procedure computed tomography (CT) scan of abdomen and pelvis showed free air, however, without gastrografin leak and successful closure of the defect with the clip (Figure 1C). Patient recovered well and was successfully discharged home on day 3.

In the second case, an 80 years old female with multiple co-morbidities underwent percutaneous endoscopic gastrostomy (PEG) placement. After successful PEG tube placement a 1 cm × 2 cm tear was seen along the lesser curvature (Figure 2A). Successful closure of the defect was achieved by deploying an OTSC 12/6 GC (Figure 2B). Post procedure CT abdomen showed free air but without extra-luminal contrast extravasation (Figure 2C). Eventually, the patient was started on regular PEG feeding and discharged to sub acute rehabilitation on day 3.

In the third case, a 67 years old female underwent colonoscopy for hematochezia. During the procedure a recto sigmoid junction perforation with an omental cover

was seen (Figure 3A). Successful closure of defect was achieved by deploying an OTSC 12/6 GC (Figure 3B). CT scan showed free air with the clip appeared to be closing the defect (Figure 3C). As an abundant measure of caution, laparoscopy was performed, which did not reveal an ongoing air leak and confirmed successful closure by OTSC placement. The area was oversown at laparoscopy in order to achieve additional approximation. Patient was further observed closely in SICU. During subsequent days, patient began improving clinically and was initially started on clears liquid diet which was advanced as tolerated. Patient was discharged home on day 5.

In summary, the ability to successfully close iatrogenic perforations with the OTSC is a significant advancement in this field. This enhances patient safety and improves outcome, especially in fragile patients. Our experience supports the promising role of OTSC system as part of the therapeutic armamentarium of the endoscopist to deal with iatrogenic gastrointestinal perforations but further studies with a large sample size will be needed to confirm its efficacy and safety. We would recommend all endoscopy units including office based and endocenters have a ready supply of clips, with proper training for physicians and staff on its use.

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P- Reviewer: Palazzo F, Tsai MH **S- Editor**: Qi Y **L- Editor**: A **E- Editor**: Wang CH

World Journal of Gastroenterology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i32.11463

World J Gastroenterol 2014 August 28; 20(32): 11463-11466
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Nonselective β-blockers may induce development of portal vein thrombosis in cirrhosis

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Received: January 4, 2014 Revised: February 13, 2014
Accepted: April 28, 2014
Published online: August 28, 2014

Abstract

Currently, nonselective β-blockers (NSBBs) are commonly used for the prevention of variceal bleeding in liver cirrhosis. The beneficial effects of NSBBs are primarily attributed to the reduction in cardiac output by blockade of β1 receptors and vasoconstriction of the splanchnic circulation by the blockade of β2 receptors. The prognostic value of occlusive portal vein thrombosis (PVT) in cirrhotic patients has been increasingly recognized. The most important risk factor for the development of PVT in liver cirrhosis is the decreased portal vein inflow velocity. Collectively, we propose that the use of NSBBs potentially increases the development of portal vein thrombosis by reducing portal vein inflow velocity. The hypothesis should be confirmed by prospective cohort studies, in which cirrhotic patients without prior PVT treated with and without NSBBs are enrolled, and the development of PVT during follow-up is compared between the two groups. Additionally, subgroup analyses should be performed according to the dosage of NSBBs and the reduction of portal inflow velocity after use of NSBBs.

Key words: Non-selective β-blockers; Propranolol; Nadolol; Portal vein thrombosis; Liver cirrhosis

Core tip: Non-selective β-blockers can reduce portal flow velocity and induce development of portal vein thrombosis in liver cirrhosis.

Qi XS, Bai M, Fan DM. Nonselective β-blockers may induce development of portal vein thrombosis in cirrhosis. *World J Gastroenterol* 2014; 20(32): 11463-11466 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i32/11463.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i32.11463

TO THE EDITOR

Benefits of non-selective β-blockers in the treatment of cirrhotic portal hypertension

Currently, benefits of non-selective β-blockers (NSBBs), such as propranolol and nadolol, are recommended as the mainstay treatment of choice for primary and secondary prevention of variceal bleeding in liver cirrhosis in different practice guidelines and consensus statements[1-3]. This recommendation primarily originates from the positive results of numerous randomized controlled trials and meta-analyses that NSBBs significantly decrease the incidence of first or recurrent variceal bleeding and improve survival in cirrhotic patients[4-9]. It appears that the benefits of NSBBs have been the "aspirin" of the hepatologists[10]. The principle mechanisms of action of NSBBs include: (1) decreasing cardiac output by blockade of β1 receptors and (2) constricting the splanchnic

circulation by the blockade of β2 receptors. These beneficial effects can reduce the portal vein inflow velocity, thereby lowering portal vein pressure[2,3]. Certainly, several inconveniences and/or inappropriateness should not be neglected. First, approximately 15% of patients may have contraindications to NSBBs[2,3]. Second, an additional 15% of patients are intolerant of NSBBs due to drug-related adverse events[2,3]. Third, only 30%-50% of patients treated with NSBBs can achieve a sufficient hemodynamic response (a reduction in hepatic venous pressure gradient ⩾ 20% from baseline level or to ⩽ 12 mmHg)[11,12]. Fourth, NSBBs may be ineffective for preventing enlargement of small varices[13].

In addition, a meta-analysis of three randomized controlled trials and three retrospective studies demonstrated that NSBBs could prevent the occurrence of spontaneous bacterial peritonitis in cirrhotic patients, regardless of hemodynamic response[14]. Further studies showed its potential mechanism, that the use of NSBBs could ameliorate gastroduodenal and intestinal permeability and reduce bacterial translocation, irrespective of the hemodynamic effect on portal hypertension[15,16].

Adverse effects of NSBBs in treatment of cirrhotic portal hypertension

Recently, a single-center, observational, case-only, prospective study by Sersté *et al*[17] demonstrated that NSBBs were significantly associated with poor survival in cirrhotic patients with refractory ascites (median survival time: 20 mo in patients without propranolol *vs* 5 mo in those with propranolol, $P = 0.0001$). Subsequently, a self-controlled crossover study by the same investigators found that the negative prognostic effect of NSBBs might be attributed to a high risk of paracentesis-induced circulatory dysfunction in cirrhotic patients with refractory ascites[18]. The milestone study strongly suggests that the use of NSBBs should be undertaken with caution in these patients. Certainly, not all studies have supported this finding. More recently, a retrospective cohort of 114 consecutive patients undergoing regular paracentesis with or without NSBBs at modest doses (36 patients *vs* 78 patients) was analyzed[19]. Median survival time was similar between the NSBB and no NSBB groups (18 mo *vs* 11 mo, $P = 0.98$, log-rank test). In addition, Galbois and colleagues showed no effect of NSBBs on the mortality of cirrhotic patients admitted to the intensive care unit due to severe sepsis or septic shock[20]. Due to many controversies among studies, the negative effect of NSBBs on cirrhotic patients deserves further exploration.

Clinical significance of portal vein thrombosis in liver cirrhosis

Accumulated evidence has confirmed that the presence of occlusive portal vein thrombosis (PVT) is negatively associated with the outcome of cirrhotic patients, by elevating the incidence of variceal bleeding and decreasing survival[21-23]. Although the resolution of partial PVT can be frequently observed[24], the role of occlusive PVT as a clinical predictor of decompensated liver cirrhosis has been hypothesized[25]. To prevent further these detrimental effects, clinicians should pay more attention to identify cirrhotic patients at high risk of the development of PVT[26].

Risk factors of PVT in liver cirrhosis

To date, several risk factors of PVT in liver cirrhosis have been established[26-28]. The most important risk factor is decreased portal inflow velocity in liver cirrhosis. In an Italian prospective observational study by Zocco *et al*[29], a total of 100 consecutive cirrhotic patients without prior PVT were followed to evaluate whether or not *de novo* PVT developed within 1 year. Demographic, clinical, biochemical, and ultrasound imaging data at baseline were compared between patients who developed *de novo* PVT and those who did not. In univariate analysis, higher Model for End-stage Liver Disease score, lower platelet count, reduced antithrombin and protein C and S levels, and decreased portal inflow velocity < 15 cm/s were associated with the development of PVT in liver cirrhosis. In multivariate analysis, the decreased portal inflow velocity was the only independent predictor of PVT. Notably, the role of inherited coagulation abnormalities (*i.e.*, factor V Leiden and prothrombin gene mutation), which might be associated with the presence of PVT in liver cirrhosis[30,31], was not analyzed in this study. More recently, another study has confirmed the relationship between reduced portal inflow velocity and the development of PVT (mean velocity portal vein flow: 9 ± 0.9 cm/s in patients who developed *de novo* PVT *vs* 12.5 ± 2.3 cm/s in those who did not, $P < 0.001$)[32].

Possible relationship between use of NSBBs and occurrence of PVT in liver cirrhosis

Considering the capability of NSBBs to reduce portal vein inflow and portal pressure, we propose that NSBBs may further induce the occurrence of PVT in liver cirrhosis. Many uncertainties exist regarding whether or not the dosage of NSBBs and the level of portal pressure or heart rate reduction by NSBBs are associated with the occurrence of PVT. Furthermore, if possible, the clinical significance of PVT induced by NSBBs on patients' survival remains uncertain.

Ideally, this hypothesis should be confirmed by prospective cohort studies, in which cirrhotic patients without prior PVT treated with and without NSBBs are followed up, and the development of PVT is evaluated. Additionally, subgroup analyses may be necessary according to the dose of NSBBs and the reduction of portal inflow velocity after NSBB treatment.

To the best of our knowledge, the relationship between NSBBs and development of PVT in liver cirrhosis has been explored in only one European Association for the Study of the Liver abstract[32], in which 56 consecutive cirrhotic patients without hepatocellular carcinoma were enrolled and evaluated about the occurrence of PVT every 6 mo during follow-up. Multivariate analysis

demonstrated that use of NSBBs was an independent predictor of developing PVT in liver cirrhosis (OR = 3.3, 95%CI: 1.4-6.8, $P < 0.001$). These results should be further validated in well-designed prospective studies with a larger sample.

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World J Gastroenterol 2014 August 28; 20(32): I-VI
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

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Name of journal
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ISSN
ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

Launch date
October 1, 1995

Frequency
Weekly

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Statistical data

Write as mean ± SD or mean ± SE.

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Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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