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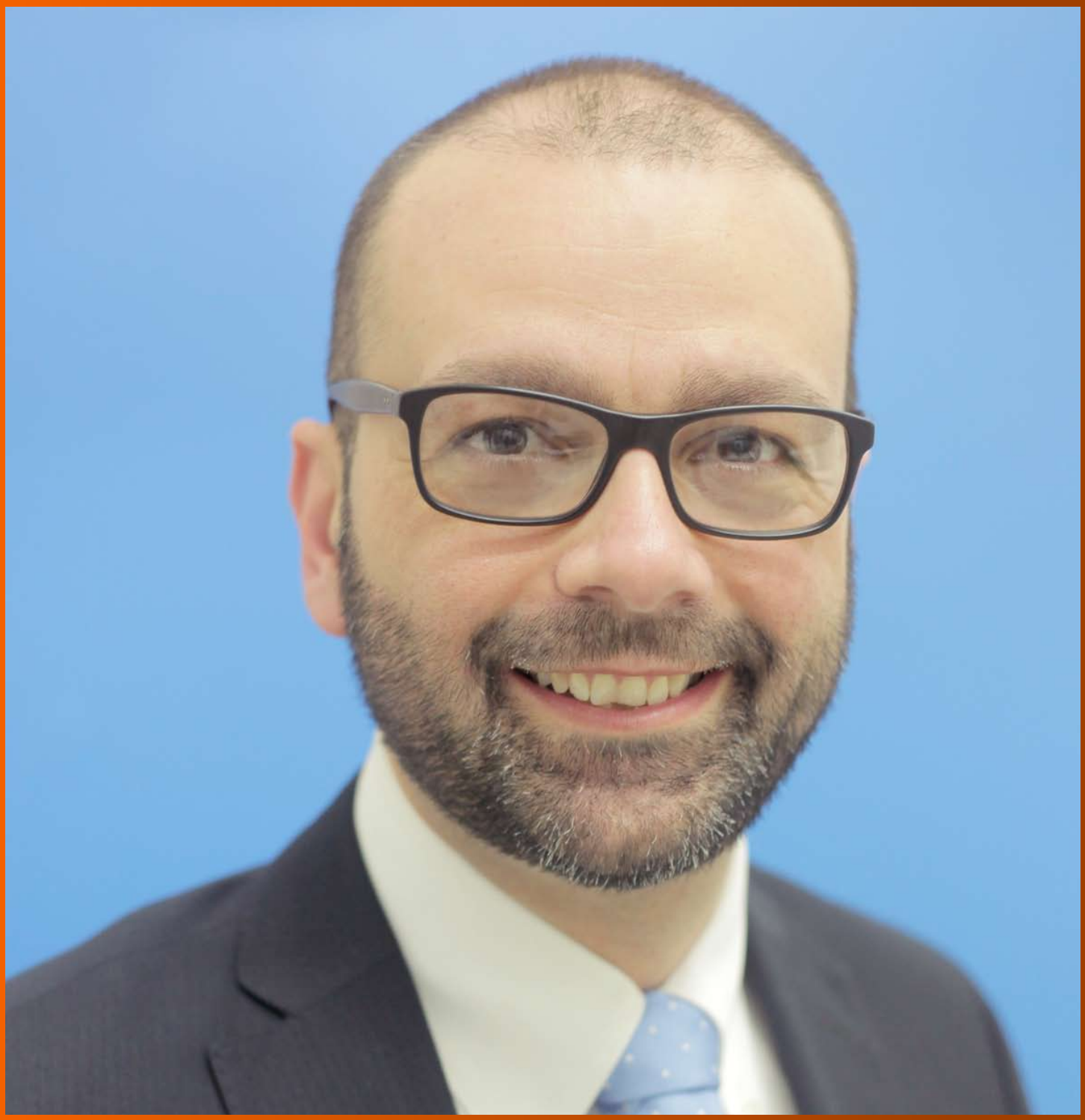

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EDITORIAL

Inflammatory pouch disease: The spectrum of pouchitis

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Abstract

Restorative proctocolectomy with ileal-pouch anal anastomosis (IPAA) is the operation of choice for medically refractory ulcerative colitis (UC), for UC with dysplasia, and for familial adenomatous polyposis (FAP). IPAA can be a treatment option for selected patients with Crohn's colitis without perianal and/or small bowel disease. The term "pouchitis" refers to nonspecific inflammation of the pouch and is a common complication in patients with IPAA; it occurs more often in UC patients than in FAP patients. This suggests that the pathogenetic background of UC may contribute significantly to the development of pouchitis. The symptoms of pouchitis are many, and can include increased bowel frequency, urgency, tenesmus, incontinence, nocturnal seepage, rectal bleeding, abdominal cramps, and pelvic discomfort. The diagnosis of pouchitis is based on the presence of symptoms together with endoscopic and histological evidence of inflammation of the pouch. However, "pouchitis" is a general term representing a wide spectrum of diseases and conditions, which can emerge in the pouch. Based on the etiology we can sub-divide pouchitis into 2 groups: idiopathic and secondary. In idiopathic pouchitis the etiology and pathogenesis are still unclear, while in secondary pouchitis there is an association with a specific causative or pathogenetic factor. Secondary pouchitis can occur in up to 30% of cases and can be classified as infectious, ischemic, non-steroidal anti-inflammatory drugs-induced, collagenous, autoimmune-associated, or Crohn's disease. Sometimes, cuffitis or irritable pouch syndrome can be misdiagnosed as pouchitis. Furthermore, idiopathic pouchitis itself can be sub-classified into types based on the clinical pattern, presentation, and responsiveness to antibiotic treatment. Treatment differs among the various forms of pouchitis. Therefore, it is important to establish the correct diagnosis in order to select the appropriate

treatment and further management. In this editorial, we present the spectrum of pouchitis and the specific features related to the diagnosis and treatment of the various forms.

Key words: Pouchitis; Idiopathic pouchitis; Secondary pouchitis; Ulcerative colitis, Crohn's disease

Core tip: Proctocolectomy with ileal-pouch and anal anastomosis is the operation of choice for refractory ulcerative colitis, for colitis with dysplasia, for familial polyposis and for selected Crohn's colitis patients. Pouchitis symptoms are non-specific, and cannot be used alone to identify the cause. Furthermore, the name "pouchitis" is a general term representing many conditions, with differing causes and treatments. To manage pouchitis appropriately, one must be able to establish the correct diagnosis. In this paper, we have reviewed the diagnostic methods, including endoscopy, histology and other investigative tools, with the goal of being able to provide the correct diagnosis and treatment.

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POUCHITIS: DEFINITIONS AND CLASSIFICATION

Restorative proctocolectomy with ileal-pouch and anal anastomosis (IPAA) is the operation of choice for refractory ulcerative colitis (UC), for UC with dysplasia, and for familial adenomatous polyposis (FAP). Additionally, IPAA can be a treatment option for a selected group of patients with Crohn's colitis without perianal and/or small bowel disease[1].

Pouchitis is a common complication in patients with IPAA, but the term is nonspecific, and encompasses a variety of etiologies and pathogenesis. Interestingly, while pouchitis may occur in up to 50% of patients with UC, it is rarely seen in patients with FAP[2,3]. This suggests that the pathogenetic background of UC may contribute significantly to the development of pouchitis.

The symptoms of pouchitis are nonspecific[4] and can include increased bowel frequency, urgency, tenesmus, incontinence, nocturnal seepage, rectal bleeding, abdominal cramps, and pelvic discomfort. Extraintestinal manifestations, involving joints, eyes, skin and liver may also be present, more commonly in UC patients with IPAA[5].

Table 1 Classification of "pouchitis" according to the etiology

Idiopathic pouchitis
Secondary pouchitis
Infectious
Bacterial pathogens
Clostridium difficile, *Campylobacter jejuni*, *Salmonella typhi*, *Escherichia coli*, *Klebsiella*, *Pseudomonas*, others
Fungi: *Candida*
Viruses: CMV
Ischemic
NSAID-associated
Collagenous
Autoimmune-associated
Crohn's disease-associated
Other diagnoses
Cuffitis
Irritable pouch syndrome

NSAID: Non-steroidal anti-inflammatory drug.

The diagnosis of pouchitis is based on the presence of symptoms plus endoscopic and histological evidence of inflammation of the pouch. In general, pouchitis can present in 3 forms - acute, relapsing or chronic.

However, "pouchitis" is a general term like "colitis", and represents a wide spectrum of diseases and conditions, which can emerge in the pouch (Table 1). Based on etiology, we can identify 2 main diagnostic pouchitis groups - idiopathic and secondary. In "idiopathic" pouchitis, the etiology and pathogenesis are unclear, while in "secondary" pouchitis, there is an association with a specific causative or pathogenetic factor[6]. Secondary pouchitis occurs in up to 30% of cases and can be infectious, ischemic, non-steroidal anti-inflammatory drug (NSAID)-induced, collagenous, autoimmune-associated, or due to Crohn's disease. Sometimes, cuffitis or irritable pouch syndrome are misdiagnosed as pouchitis. Furthermore, idiopathic pouchitis can be sub-classified in types based on the clinical pattern, presentation, and responsiveness to antibiotic treatment.

IDIOPATHIC POUCHITIS

In the majority of patients with pouchitis, the etiology and pathogenesis are not clear and the disease is identified as idiopathic pouchitis. The pathogenesis of pouchitis in these patients may be triggered by dysbiosis, leading to an altered mucosal immune response.

Pathogenesis

The intestinal microbiota, the intestinal epithelial cells and the immune system of the gut epithelium play a vital role in pouch homeostasis[7]. Following ileostomy closure, the ileal mucosa of the pouch is exposed to feces containing higher bacterial concentrations than in the ileum of a healthy individual; this is due to relative fecal stasis in the pouch[7]. During the first year following ileostomy closure, adaptive changes occur

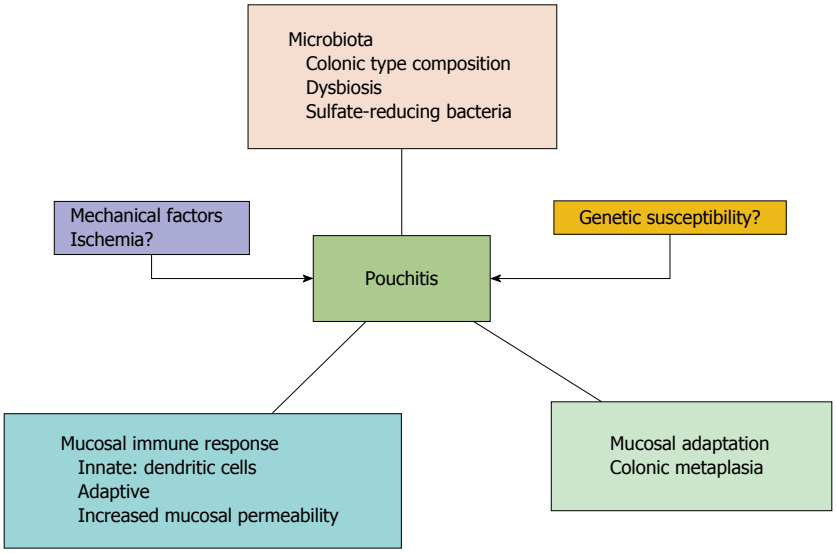

Figure 1 Idiopathic pouchitis pathogenesis.

gradually in the pouch microbiota and mucosa. The pouch microbiota shift to a colon-like composition[8] and colonic metaplasia of the pouch mucosa occurs, where mucosal goblet cells and columnar epithelial cells acquire colonic morphologic and functional characteristics[9]. Current evidence suggests that the interactions of the altered composition and/or the quantity of the luminal microbiota (dysbiosis), with the altered characteristics of the mucosa (colonic metaplasia), in conjunction with abnormalities of innate and adaptive mucosal immunity, play a key role in the pathogenesis of pouchitis (Figure 1)[7,10,11].

Microbiota: Pouchitis in UC patients is usually responsive to antibiotic therapy, which is presumed to indicate that bacteria are involved in the pathogenesis. However, microbiological investigations of the bacterial communities with cultures of stool or mucosal biopsies have failed to reveal the culprit species. Furthermore, even the new molecular microbiological techniques have failed to identify a certain species or a group of species which can specifically be associated with pouchitis in patients with either a UC or FAP pouch[12]. However, studies have shown that pouch microbiota differ between UC patients and FAP patients[13]. Bacterial diversity seems to play a protective role since it is significantly greater in FAP patients compared to UC patients without pouchitis, and it was also greater in UC patients without pouchitis compared to UC patients with pouchitis[12]. In addition, there may be a temporary alteration in the composition of the microbial community during pouchitis compared to the non-inflamed pouch, both in UC and FAP pouch[13]. Sulfate-reducing bacteria may play a role in UC patients with pouchitis. These bacteria exclusively colonize pouches in UC patients and not in FAP patients; the reasons are unknown[14]. Since idiopathic pouchitis is seen mostly in UC patients, these bacteria may well play a role in pouchitis pathogenesis.

Immune responses: The interactions between the pouch microbiota and host immune responses, innate and adaptive, play also significant role in pouchitis pathogenesis. Innate mucosal immune reactions are implicated in pouchitis pathogenesis, while the adaptive mucosal immunity reactions probably are an epi-phenomenon after the activation of a nonspecific inflammatory cascade or pathway[15,16]. Mucosal barrier dysfunction, with increased intestinal permeability, is also believed to play a role in pouchitis[17]. In a recent study, Landy *et al*[18] found abnormalities in tight junction protein (TJP) expression in UC pouches and differences in dendritic cell (DC) expression of gut-homing markers and toll-like receptors (TLRs) between the inflamed and non-inflamed ileal pouch in patients with UC, but not FAP.

Genetics: So far, only a few small studies have analyzed genetic susceptibility to pouchitis. Polymorphisms in the interleukin-1 receptor antagonist gene allele 2, tumor necrosis factor (TNF) allele 2, TLR 1 and NOD2/CARD15 have been associated with pouchitis. In addition, the carriers of the toll-like receptor (TLR) 9-1237C and the CD14-260T alleles were found significantly more often to have the chronic relapsing form of pouchitis[11].

Risk factors

Reported risk factors for pouchitis include genetic susceptibility (polymorphisms of IL-1ra and NOD2/CARD15, non-carrier status of TNF allele 2), extensive UC, backwash ileitis, preoperative thrombocytosis or corticosteroid use, extraintestinal manifestations, especially PSC, the presence of p-ANCA, non-smoking status, and the use of NSAIDs[10]. Different risk factors may be associated with the different types of

Table 2 The variety of idiopathic pouchitis classifications

Activity
Active
Inactive
Presentation
Acute < 4 wk duration
Chronic > 4 wk duration
Clinical pattern
Single episode
Infrequent < 4 episodes a year
Relapsing > 4 episodes a year
Continuous
Response to treatment
Responsive
Refractory
Response to antibiotics
Antibiotic-responsive:
Infrequent episodes (< 4 episodes per year) responding to a 2-wk course of a single antibiotic
Antibiotic-dependent
Frequent episodes or persistent episodes of pouchitis requiring long-term, continuous therapy for maintaining remission
Chronic antibiotic-refractory
Not responding to a 4-wk course of metronidazole or ciprofloxacin, requiring prolonged therapy of ≥ 4 wk consisting of 2 or more antibiotics, oral or topical 5-ASA, corticosteroids, AZA/6-MP, or biologics

pouchitis, suggesting different pathogenic pathways in the various forms of idiopathic pouchitis and secondary pouchitis.

Classification

Idiopathic pouchitis can be categorized as acute, acute relapsing, or chronic. It can also be classified as antibiotic-responsive, antibiotic-dependent and antibiotic-refractory (Table 2)[10,19]. It is important to emphasize that approximately 20%-30% of patients with chronic antibiotic-refractory pouchitis are misclassified, and actually have secondary pouchitis. The management of these conditions differs from that for idiopathic pouchitis and is specific to the underlying etiology.

SECONDARY POUCHITIS

Clostridium difficile pouchitis

Clostridium difficile (*C. difficile*) infection (CDI) is a common cause of diarrhea in hospitalized patients, including those with IBD[20]. It is now also a common cause of antibiotic-associated diarrhea in the general population with increasing incidence[21]. CDI can also affect IBD patients post-surgically, both in ileostomates, with increased stoma output, and in patients with IPAA, in whom CDI may range from simple asymptomatic colonization to chronic antibiotic-refractory pouchitis[22,23]. Fulminant Clostridium difficile-associated pouchitis has also been described[24]. Management choices should reflect standard practices for the treatment of this infection.

Infectious pouchitis

In a recent study, bacterial pathogens other than *C. difficile* have been identified in some patients with chronic refractory pouchitis[25]. Fecal samples were analyzed in 15 patients with active refractory pouchitis and the cultures revealed *Escherichia coli, Klebsiella, unclassifiable coliforms, Pseudomonas*, and *Morganella* in isolation or in combination. Treatment was based on antibiotic sensitivity results; clinical response and remission was achieved in 12 out of 15 cases (80%). This study showed that fecal culture, fecal coliform sensitivity testing and targeted antibiotic treatment can be beneficial in some patients with refractory pouchitis. It is important to notify the lab to perform sensitivities on all predominant organisms, and to not discard cultures of what appear to be commensals.

Candidal pouchitis

Although fungal pouchitis as a distinct form of pouchitis has not yet been described, fungal infection might be involved in a subgroup of patients with chronic refractory pouchitis. Navaneethan *et al*[6] reported that they have occasionally seen pouchitis in the setting of systemic candidiasis, although fungal invasion of the pouch tissue on histology was rare. In addition, they mention that clotrimazole has been shown to benefit patients with refractory pouchitis who had previously failed to respond to standard antibiotic therapies[6]. Although there is as yet no completed study, the authors stated that a study was in progress assessing the effectiveness and safety of topical clotrimazole enema in pediatric and adult patients with pouchitis (http://clinicaltrials.gov/ct2/show/NCT00061282)[6].

CMV pouchitis

CMV infection in patients with IPAA can cause chronic pouchitis with a clinical presentation similar to idiopathic pouchitis, with the only difference being that patients with CMV-associated pouchitis more

often have fever compared to those with idiopathic pouchitis[26].

Ischemic pouchitis: Pouch ischemia may also be a cause of pouchitis. Characteristically, ischemic pouchitis is more often found in the efferent limb of the pouch[27]. Factors related to the surgical construction of the pouch have been implicated, including disruption of the vessels supplying the distal ileum during colectomy or the tension of the mesentery and/or the vessels that supply the distal ileum during the IPAA construction. However, besides the mechanical factors, the underlying disease may also play role, since ischemic pouchitis is more common in UC patients than in those with FAP[28]. Ischemic pouchitis may also be related to oxidative stress of the endothelial cells, due to ischemia-reperfusion injury, which eventually results in inflammation of the pouch mucosa[29]. Patients with IPAA have lower plasma concentrations of lipophilic antioxidants (alpha-carotene, beta-carotene and lycopene) and higher free radical activity suggesting increased oxidative stress[29]. Patients with ischemic pouchitis are often mis-classified as having chronic antibiotic-refractory pouchitis. Most of these patients have minimal symptoms, and do not require management.

NSAID-induced pouchitis: It is well known that NSAID use can induce mucosal injury in the GI tract and can exacerbate disease activity in IBD patients. Not surprisingly, in a subset of patients with IPAA, NSAIDs can cause erosions in the pouch mucosa, which can result in either a pure NSAID-induced pouchitis *per se* or exacerbation of a pre-existing idiopathic pouchitis. NSAID use should always be elucidated in patients with chronic antibiotic-refractory pouchitis[30]. Elimination of NSAIDs should result in resolution.

Autoimmune pouchitis: In a subgroup of patients with antibiotic-refractory chronic pouchitis, there is emerging evidence implicating autoimmunity as a factor. In these patients the pouchitis has some particular features, including: no response to conventional antibiotics; presence of extraintestinal manifestations such as arthralgia or PSC; concurrent autoimmune disorders such as asthma, psoriasis, type I diabetes, rheumatoid arthritis, autoimmune thyroid diseases, psoriasis, systemic lupus erythematosus; presence of serum autoantibodies (pANCA); and responsiveness to immunosuppressive therapies (steroids, thiopurines, biologics)[5].

Autoimmune pouchitis includes the PSC-associated and IgG4-associated forms of pouchitis. PSC has been described as a risk factor for the development of pouchitis in UC patients with IPAA. PSC-associated pouchitis predisposes to chronic antibiotic-resistant pouchitis. IgG4-associated pouchitis represents another subgroup of autoimmune pouchitis that predisposes to a more severe chronic antibiotic-resistant pouchitis. It is characterized by elevated serum IgG4 and/or infiltration of the pouch mucosa with IgG4-expressing plasma cells, even in the absence of concurrent autoimmune pancreatic disease[5].

Crohn's pouchitis: Crohn's disease of the pouch can occur in patients with prior Crohn's colitis without previous small intestinal or perianal disease. More interestingly, Crohn's disease of the pouch can develop *de novo* in UC patients after colectomy with IPAA. CD of the pouch can exhibit the inflammatory, fistulizing or fibrostenotic phenotypes of classic CD and can affect any part of the gastrointestinal tract including the proximal GI tract, the neo-terminal ileum, the pouch and the perianal area. CD pouchitis is a more complex disease than the idiopathic variant or the other secondary forms of pouchitis. Separation of this diagnosis from the other forms of pouchitis, as well as from the surgery-associated complications is required[31]. With a view to pouch preservation, management should be relatively aggressive; unless antibiotics are highly effective, immunomodulators and/or biologics should be used, with the goal of mucosal healing.

Cuffitis: Cuffitis refers to inflammation of the rectal cuff in the area between the anastomosis and dentate line. Cuffitis may be a variant of UC or simply represent a flare of UC in the rectal cuff, and is particularly common in IPAA constructed with stapled anastomosis without mucosectomy. With this technique a 1-2 cm segment of rectal columnar epithelium remains *in situ*, increasing the risk for cuffitis and requiring surveillance for dysplasia. The symptoms of cuffitis can be very similar to those of pouchitis plus the presence of bloody bowel movements. Thus, differential diagnosis from pouchitis is required since the management can be different[32-34].

Irritable pouch syndrome: The irritable pouch syndrome is a functional disorder of unclear cause in patients with IPAA. The patients present with symptoms of pouchitis without endoscopic or histologic evidence of inflammation in the pouch mucosa. It is a diagnosis of exclusion; aside from idiopathic and secondary pouchitis, other diagnoses which should be excluded include celiac disease, lactose or fructose intolerance, and proximal small-bowel bacterial overgrowth, and possibly others[35].

DIAGNOSIS AND DIFFERENTIAL DIAGNOSIS

"Pouchitis" represents a wide spectrum of inflammatory and non-inflammatory disorders of the pouch, with different pathogenetic mechanisms, presentations, courses, prognoses and treatments. It is

Table 3 The pouchitis disease activity index

Criteria	Score
Clinical	
Stool frequency	
Usual postoperative stool frequency	0
1-2 stool/d > postoperative usual	1
3 or more stool/d > postoperative usual	2
Rectal bleeding	
None or rare	0
Present daily	1
Fecal urgency or abdominal cramps	
None	0
Occasional	1
Usual	2
Fever (temperature > 37.8 ℃)	
Absent	0
Present	1
Endoscopic findings	
Edema	1
Granularity	1
Friability	1
Loss of vascular pattern	1
Mucous exudates	1
Ulceration	1
Histological findings - acute histological inflammation	
Polymorphonuclear leucocyte infiltration	
Mild	1
Moderate without crypt abscess	2
Severe with crypt abscess	3
Ulceration per low-power field (mean)	
< 25%/25%-50%/> 50%	1/2/3
Total pouchitis disease activity index (max 18) pouchitis ⩾ 7	

Table 4 The pouchitis activity score

Criteria	Score
Clinical	
Stool frequency/24 h: < 8/8-10/10-13/> 13	0/2/4/6
Urgency: absent/present	0/3
Rectal bleeding: absent/present	0/3
Endoscopic findings	
Edema: absent/present	0/1
Granularity: absent/present	0/1
Friability: absent/mild/severe	0/1/2
Erythema: absent/mild/severe	0/2/3
Mucosal flattening: absent/present	0/2
Ulcerations/erosions: absent/mild/severe	0/2/3
Histological findings	
Acute histological inflammation	
Polymorphonuclear leucocyte infiltration absent/discrete and patchy/moderate ± crypt abscesses or cryptitis/ extensive ± crypt abscesses or cryptitis	0/1/2/3
Ulcerations/erosions absent/mild and superficial/moderate/extensive	0/1/2/3
Chronic histological inflammation	
Polymorphonuclear leucocyte infiltration absent/mild and patchy/moderate/extensive	0/1/2/3
Villous atrophy absent/minimal/partial/subtotal-total	0/1/2/3
Total pouchitis activity score (max 36)	
Pouchitis ⩾ 13	

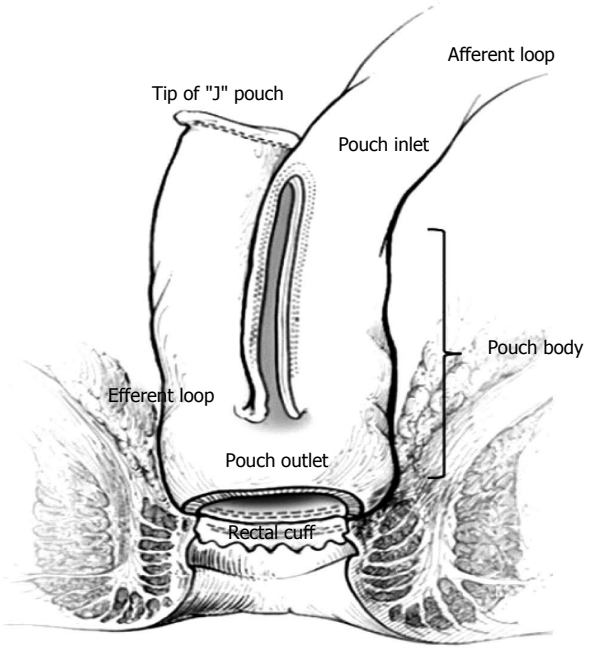

Figure 2 The J-pouch anatomy (adapted and modified from Cima *et al*[40]).

therefore important to establish the correct diagnosis in order to optimize the management and treatment. The symptoms of pouchitis are non- specific. As is often the case in IBD, the severity of the symptoms does not necessarily correlate with the severity of endoscopic and/or histologic findings[36]. It is generally accepted that the diagnosis and differential diagnosis of pouchitis should be based on a combination of clinical, endoscopic and histological findings[37].

Two scoring systems have been developed to diagnose pouchitis and to assess disease severity, the pouchitis disease activity index[38] and the pouchitis activity score (PAS)[39]. Both combine the scoring of clinical symptoms, endoscopic findings and histologic features (Tables 3 and 4) and are commonly used in clinical trials. On the other hand, in clinical practice, endoscopy is the most accurate and valuable tool to diagnose the presence, describe the features and assess the severity of inflammation in the pouch mucosa.

Pouchoscopy guides the next steps of the diagnostic work-up and, finally, the treatment. During pouchoscopy it is important to identify and carefully evaluate the pouch outlet, the pouch body, the efferent limb, the tip of the pouch (in J pouch), the pouch inlet, the afferent limb, the staple lines and the anal transitional zone or cuff (Figure 2)[40]. The endoscopist should collect information about the anatomical construction of the pouch and the severity, extent, and distribution of mucosal inflammation in the various parts of the pouch. The presence of edema, granularity, erythema, friability, spontaneous bleeding, erosions and ulcerations should be recorded[41]. Additionally, one should record the presence of afferent loop ileitis, cuffitis, or inflammatory polyps.

Although one might expect a totally normal mucosa in the pouch, mild patchy edema and erythema are

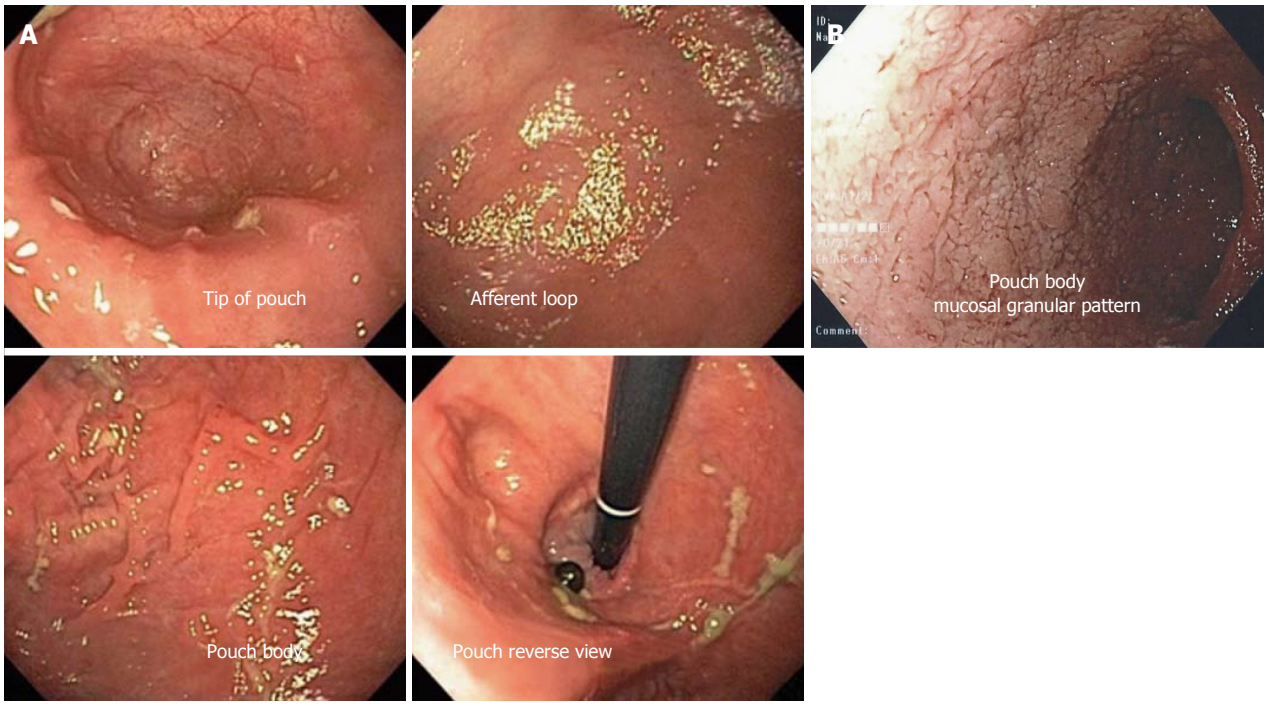

Figure 3 Normal pouch mucosa (A), mucosal granular pattern (edema) with disappearance of the mucosal vascular pattern (B).

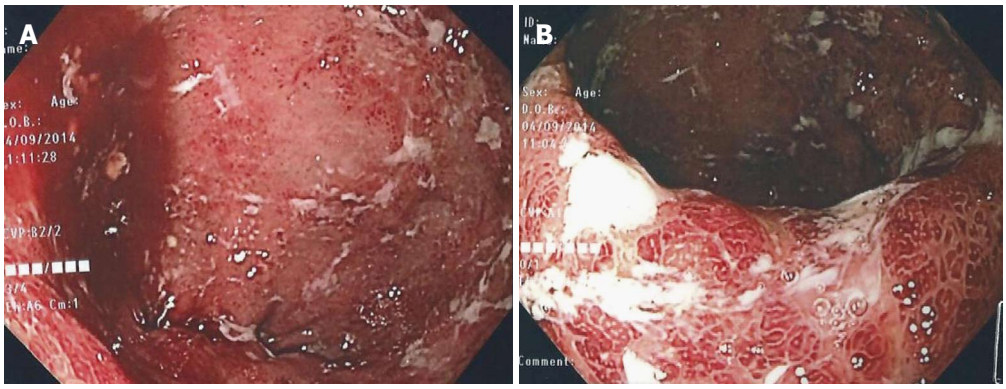

Figure 4 Active pouchitis (A, B). The mucosal vascular pattern has been lost and the mucosa is characterized by diffuse redness, severe edema with erosions and ulcers.

considered to be acceptable in a "normal" pouch (Figure 3A and B)[42]. A normal-appearing mucosa in a symptomatic patient should raise the suspicion of irritable pouch syndrome. In active pouchitis, a spectrum of findings occurs. Most typical is a mucosa with findings resembling those of active ulcerative colitis (Figure 4A and B).

Patients with CDI pouchitis typically lack the classical endoscopic or histologic features of pseudomembranes[43]. Immune-mediated pouchitis, including the PSC-associated and IgG4-associated forms, often produces diffuse inflammation in the pouch body, together with a long segment of inflammation in the afferent limb[44].

Ischemic pouchitis is characterized by an asymmetric distribution of the inflammation in the pouch body. The pouchitis is present in the efferent limb only, sparing the afferent limb of the pouch, with a sharp demarcation of inflamed and non-inflamed parts of the pouch body[27].

Patients with typical CD of the pouch may have: segmental inflammation of the pouch body and/or afferent limb[45]; strictures at the pouch inlet/outlet or in the afferent limb (Figure 5A-H); and the presence of 1 or more fistulas (perianal, pouch-vaginal or pouch-vesical)[46].

With a finding of afferent limb ileitis, one must consider the following possibilities: NSAID-induced ileitis, CD ileitis, and immune-related ileitis. Immune ileitis is continuous, whereas the lesions in NSAIDs or CD ileitis can be patchy/segmental and often extend to the distal neo-terminal ileum (more than 10 cm beyond the pouch inlet, Figure 5). On endoscopy, cuffitis is characterized by inflammation of the rectal

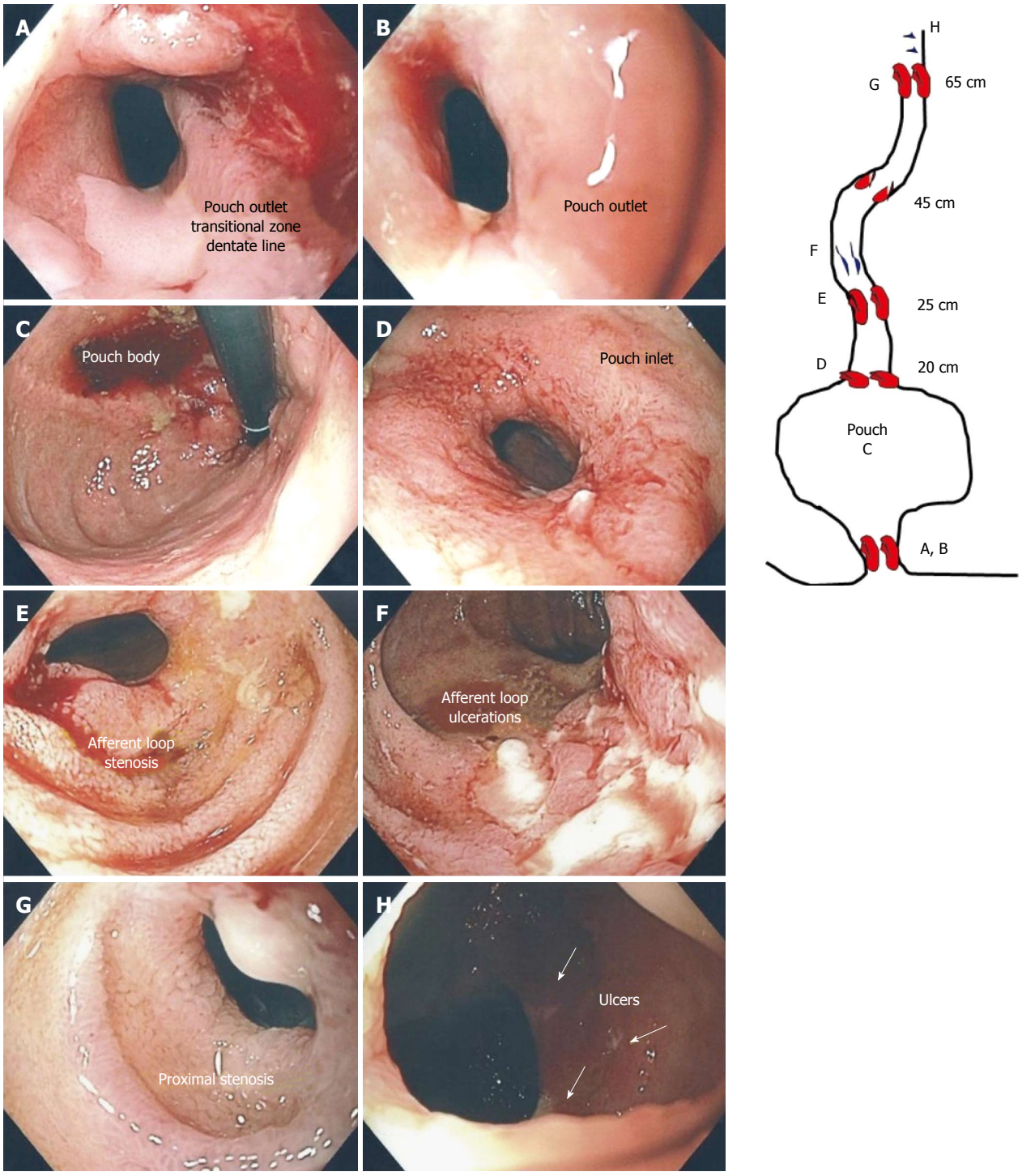

Figure 5 Crohn's ileo-pouchitis. Stenosis of the pouch outlet (A, B), normal appearing mucosa of the pouch (C), stenosis of the pouch inlet (D), stenosis of the afferent limb at 25 cm (E), linear deep ulcerations in the ileum (F), proximal stenosis of the ileum at 65 cm (G) and ulcers more proximally (H).

cuff only, while the pouch should be normal or near-normal, with minimal inflammation[34].

Histology, abdominal and pelvic imaging, stool examination and cultures, and serology can also contribute to the differential diagnosis, especially in chronic antibiotic-refractory pouchitis. Infectious causes can be excluded with stool culture and Clostridium difficile toxin assay. Antibiotic sensitivity testing in stool cultures can help identify effective antibiotics. The serology panel may include pANCA, celiac tests and serum IgG4 levels.

Histology can help by characterizing the inflammation as acute or chronic, and by providing information useful for the differential diagnosis. However, it is important to recognize that there is a default "physiologic" inflammation in the pouch mucosa that represents an adaptive response of the ileal mucosa to the pouch construction and the environment (fecal stasis). The

histologic features of the "normal" pouch include villous atrophy and crypt-cell hyperplasia, mild acute and chronic inflammatory infiltration by neutrophils, eosinophils, lymphocytes, plasma cells, and histiocytes, and colonic metaplasia of the mucosa with increased numbers of Paneth and goblet cells. True pouchitis is associated with increased villous atrophy, acute and/or chronic inflammatory infiltrates, crypt abscesses, and ulceration[47].

Specific histological features often help with the differential diagnosis. The presence of granulomas is typically indicative of CD, while viral inclusion bodies provide evidence for CMV infection, which can be confirmed with immunostaining for CMV antigen or tissue PCR for CMV, confirming the diagnosis of CMV pouchitis[26,48]. Pyloric metaplasia is a sign of chronic mucosal inflammation which can be associated with chronic antibiotic-refractory pouchitis or CD pouchitis[49,50]. Increased crypt apoptosis and lamina propria infiltration with IgG4 (+) plasma cells are observed in autoimmune-pouchitis[51,52]. In ischemic pouchitis a characteristic feature on histology is the presence of extracellular hemosiderin or hematoidin pigment deposits, while the classic histologic features of ischemic enteritis are not always present[27].

Abdominal imaging is a valuable tool for the diagnosis and differential diagnosis of ileal pouch disorders, particularly when CD is the cause. Computed tomography (CT) enterography and magnetic resonance imaging (MRI) enterography are useful for the evaluation of the location, number, and degree of strictures, the presence of abscesses, or, simply, the presence of inflammation of the pouch and the proximal small bowel. Contrast pelvic MRI or anorectal ultrasound can be used for the evaluation of the anatomy and abnormalities around the pouch body and the anal transitional zone, such as fistulas, sinus tracts, and abscesses[53].

MANAGEMENT

Antibiotics are the first-line treatment for idiopathic pouchitis. In patients with IPAA and a first attack of acute pouchitis, a course of empiric antibiotic treatment with metronidazole or ciprofloxacin can be justified, without the need of endoscopy and biopsy, since the majority of these patients will have a rapid favorable response. In approximately 40% of cases, acute pouchitis will present as a single episode without recurrence[54]. However, in 60% of the patients, acute pouchitis will follow a relapsing course after the first episode, and 20%-30% of them will develop a frequently relapsing form or refractory pouchitis[54,55].

In general, when symptoms of pouchitis appear in patients with IPAA, it is recommended, if possible, that endoscopy with biopsies be performed to establish the diagnosis, before initiating treatment. If endoscopy must be delayed, empiric treatment with antibiotics can be initiated. Stool culture and testing for *C. difficile* toxin should be obtained. In more chronic forms of pouchitis, relapsing or refractory, along with endoscopy and biopsies, the diagnostic evaluation should be expanded to cover the diagnosis and differential diagnoses of secondary or other causes of "pouchitis" (Figure 6). Subsequently, the treatment will be tailored to the specific diagnosis (Figure 7).

PREVENTION AND TREATMENT

Primary prophylaxis of a first episode of pouchitis

The prevention of pouchitis begins in the operative room during surgical construction of the pouch. A suitable-sized, not too long pouch, is less susceptible to pouchitis[56]. Excessive weight gain postoperatively has been associated with an increased risk for worse pouch outcomes, including pouchitis[57,58]. Moreover, the increase in fruit consumption and intake of antioxidants, vitamin A, and vitamin C may protect from pouchitis[59]. The use of probiotics, *i.e.*, VSL#3, has been shown to be beneficial in the primary prevention of pouchitis[60]. The administration of Lactobacillus rhamnosus GG has also shown to be effective in the primary prophylaxis[61]. However, these treatments are expensive and the long-term benefit or safety is as yet unknown[58].

Treatment of acute idiopathic pouchitis

Patients with a first episode of acute pouchitis typically respond rapidly to antibiotic therapy. Metronidazole, ciprofloxacin, tinidazole, and rifaximin have all been used in the treatment of acute pouchitis in clinical practice[62]. First-line therapy includes a 2-wk treatment with metronidazole (15-20 mg/kg per day) or ciprofloxacin (1 g/d). High-dose VSL#3 has been reported to be effective for treating mild acute pouchitis[62,63]. It is noteworthy that patients who experience pouchitis symptoms immediately post-IPAA and do not respond to the antibiotic therapy, surgery-associated complications, such as pouch anastomotic leaks, should be suspected[19].

Secondary prophylaxis of subsequent episodes of pouchitis

Relapse of pouchitis or recurrent pouchitis is common (60%) after treatment and resolution of the initial episode, and some of the patients will develop treatment-refractory disease. Long-term administration of the probiotic VSL#3 has been shown to be effective in maintaining antibiotic-induced pouchitis remission in 85% of treated patients in a 9-mo period[62,64]. However, other studies have failed to confirm this beneficial effect of VSL#3[65]. Rifaximin may be an alternative maintenance treatment[66].

Treatment of chronic antibiotic-refractory pouchitis

Chronic antibiotic-refractory pouchitis may respond

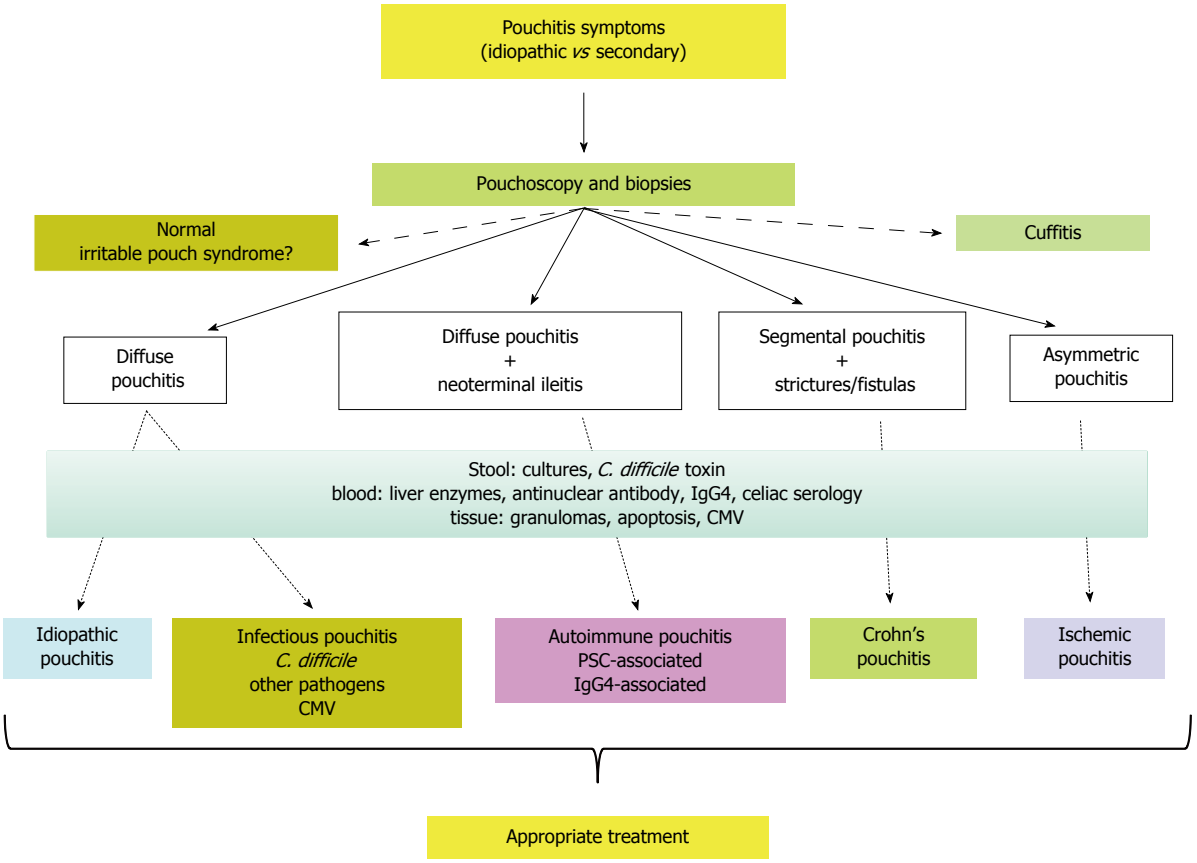

Figure 6 Pouchitis diagnostic algorithm (adapted and modified from Shen[15]). *C. difficile*: *Clostridium difficile*.

to longer courses of antibiotic combinations such as ciprofloxacin and rifaximin[67], ciprofloxacin and metronidazole[68] or ciprofloxacin and tinidazole[69]. In patients with antibiotic-resistant pouchitis, a thorough investigation is recommended. Fecal cultures and antibiotic sensitivity testing may be needed to choose the appropriate effective antibiotics. Additionally, the investigation should aim to identify and treat causes of secondary pouchitis.

Despite the atypical endoscopic findings of *C. difficile* infection in pouch patients, management choices should reflect standard practices for the treatment of this infection[70,71]. Finally, recent studies have suggested that fecal microbiota transplantation might be an alternative or adjunctive treatment for refractory CDI pouchitis[72]. CMV-pouchitis can be treated with oral or intravenous anti-CMV agents (ganciclovir)[26]. Management should reflect current practice.

If NSAID-associated pouchitis is suspected, a trial of discontinuation of NSAIDs is recommended, and should result in prompt resolution. The safety of selective COX-2 inhibitors in IPAA patients is still unknown and if those suffering from arthralgias do not respond to acetaminophen (paracetamol), they can be tried on sulfasalazine[73].

Ischemic pouchitis may be treated with allopurinol. Allopurinol is a scavenger of oxygen-derived free radicals and previous studies have shown its beneficial effect both in an animal model and in treating active pouchitis[74].

Autoimmune pouchitis, both PSC- and IgG4-associated, can be treated with corticosteroids (prednisone or budesonide), immunomodulators (azathioprine, 6-mercaptopurine), or anti-TNF biologics (infliximab, adalimumab, others). The same treatment options apply for Crohn's pouchitis together with endoscopic or surgical interventions for fistulizing or stenosing disease[75,76]. With a view to pouch preservation, management should be relatively aggressive; unless antibiotics are highly effective, immunomodulators and/or biologics should be used, with the goal of mucosal healing.

Cuffitis can be treated similarly to ulcerative proctitis with topical 5-ASA or topical steroids; suppositories should be sufficient. Cuffitis refractory to topical treatment should raise the suspicion of other conditions in the perianal and peri-pouch area. Therefore, investigation with the appropriate additional imaging studies is recommended to rule out surgical complications or CD manifestations including fistulas, sinus tracts or abscesses[77].

Finally, the treatment of irritable pouch syndrome is empiric similar to the irritable bowel syndrome, and therapies can include dietary modifications, anti-diarrheals, antispasmodics, and tricyclic antidepressants.

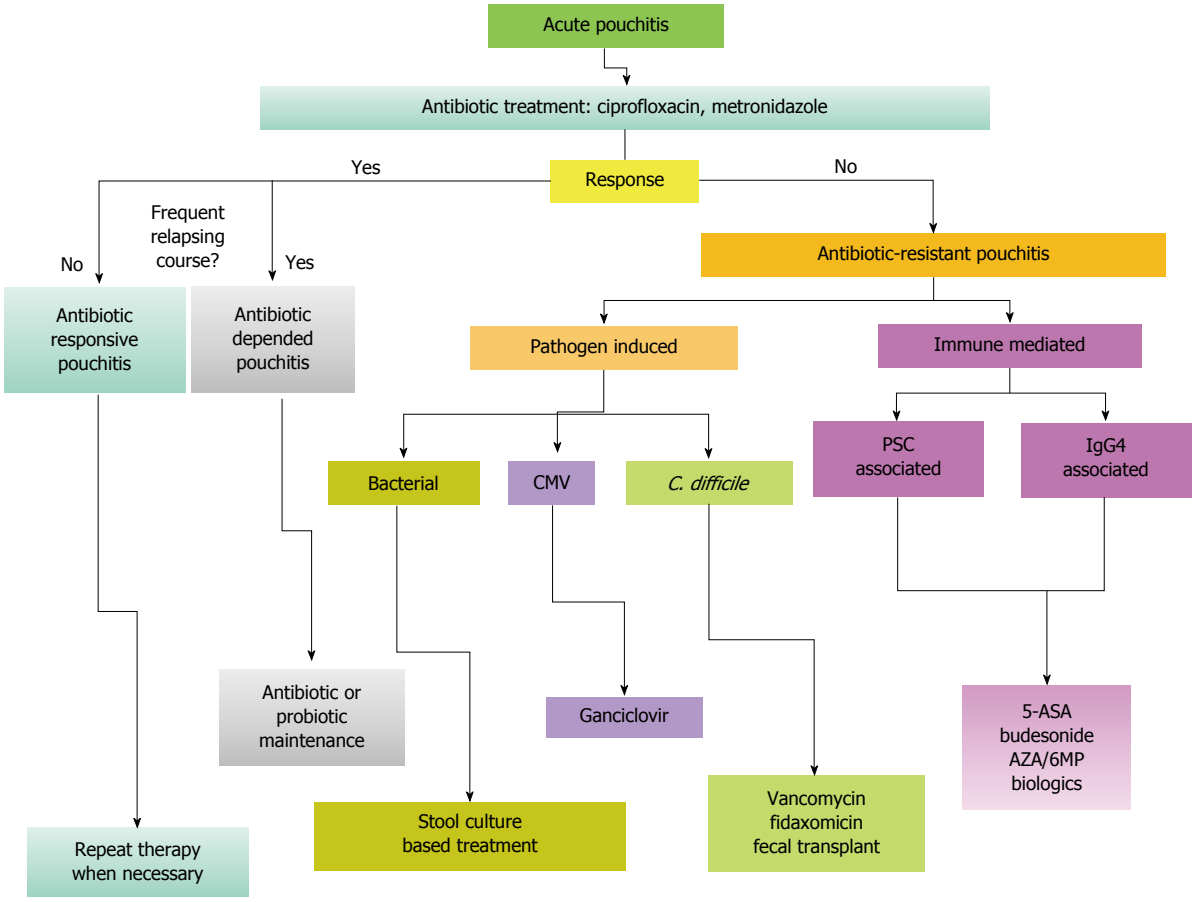

Figure 7 Pouchitis treatment algorithm (adapted and modified from Shen *et al*[15]). *C. difficile*: *Clostridium difficile*.

FUTURE TRENDS AND RESEARCH

The pouch itself and pouchitis, both acute and chronic, can serve as models to study IBD pathogenesis. Despite some differences, there are many similarities between idiopathic pouchitis and ulcerative colitis. Therefore, by studying the changes in the pouch environment that might eventually lead to pouchitis we can extract information that may help us to understand the pathogenesis of UC, as it proceeds from normal mucosa to chronic inflammation.

So far, there are no reliable animal models of pouch/pouchitis. There are technical difficulties in the creation of a pouch and translational difficulties in interpreting the results into clinical practise due to differences in the genetic background, the bacterial flora and the gut inflammation between animals and humans. On the other hand, in the human pouch model these obstacles are lacking. Pouchitis can serve as an excellent clinical model to study the pathogenetic pathways of UC, CD and other conditions that affect the bowel including *C. difficile*, CMV or NSAIDs[78].

Undoubtedly, the gut microbiome has an important role in both IBD in general and in pouchitis pathogenesis. The development of new techniques for identifying the luminal and mucosal bacterial compositions in the gut before and after pouch construction, as well as potential pathogens involved in pouchitis will contribute to understanding the IBD and pouchitis pathogenesis. The pouch may be an ideal environment to study the gut microbiota and the role of dysbiosis in IBD pathogenesis[79].

Finally, in a recent study[80] more than half of asymptomatic patients with IPAA had abnormal endoscopic and/or histologic findings on surveillance pouchoscopy, suggesting that even asymptomatic pouch patients should have pouchoscopy at regular intervals in order to diagnose and consider treating sub-clinical pouch complications at an early stage.

CONCLUSION

Pouchitis represents a disease spectrum with differences in pathogenesis, clinical manifestations, course and treatment. It is therefore important for the clinician to be aware of the various phenotypes of pouchitis and to be familiar with the clinical, endoscopic and histologic features of each one in order to plan the appropriate management and apply the suitable treatment. Pouchoscopy is the best way to assess the patient with pouchitis symptoms in order to reach the correct diagnosis, treat accordingly, and follow the course. It is important to realize that one third of chronic treatment-refractory pouchitis cases

are related to secondary etiologies or triggering factors that can be treated or modified, thus altering the course of pouchitis and avoiding pouch failure.

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TOPIC HIGHLIGHT

2015 Advances in Liver Transplantation

Neoplastic disease after liver transplantation: Focus on *de novo* neoplasms

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Abstract

De novo neoplasms account for almost 30% of deaths 10 years after liver transplantation and are the most common cause of mortality in patients surviving at least 1 year after transplant. The risk of malignancy is two to four times higher in transplant recipients than in an age- and sex-matched population, and cancer is expected to surpass cardiovascular complications as the primary cause of death in transplanted patients within the next 2 decades. Since exposure to immunosuppression is associated with an increased frequency of developing neoplasm, long-term immunosuppression should be therefore minimized. Promising results in the prevention of hepatocellular carcinoma (HCC) recurrence have been reported with the use of mTOR inhibitors including everolimus and sirolimus and the ongoing open-label prospective randomized controlled SILVER. Study will provide more information on whether sirolimus-containing *vs* mTOR-inhibitor-free immunosuppression is more efficacious in reducing HCC recurrence.

Key words: Liver transplantation; *De novo* neoplasms; Immunosuppression; mTOR inhibitors; Hepatocellular carcinoma

Core tip: With the notable increase in life expectancy after liver transplantation, together with the lengthy exposure to immunosuppression, transplant recipients are at risk of developing neoplastic disease, which accounts for almost 30% of deaths 10 years after liver transplantation. The risk of malignancy is two to four times higher in transplant recipients than in an age-

and sex-matched population, and cancer is expected to surpass cardiovascular complications as the primary cause of death in transplanted patients within the next 2 decades, making this an important topic for clinicians to consider.

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INTRODUCTION

With excellent long-term survival rates, the causes of morbidity and mortality of liver transplant (LT) recipients are primarily cardiovascular diseases, renal insufficiency, and *de novo* neoplasm, the latter of which account for almost 30% of deaths at 10 years post transplantation. Apart from hepatic causes, neoplasm has been reported as the most common cause of death in patients surviving at least 1 year after LT, and is responsible for approximately 40% of deaths[1,2]. Overall, it is estimated that in LT recipients the incidence of neoplasms is between 3.1% and 14.4%, and the cancer-related mortality rate is between 0.6% and 8.0%[3,4].

Although the risk of some neoplasms including breast cancer (1.9 times lower) and genitourinary cancer (1.5 times lower) in women seem to be reduced compared to those of the general population[5], in general terms, the status of transplant recipient is associated with an increased risk of developing *de novo* neoplasm. As shown in a study analyzing 1000 consecutive LT recipients in Pittsburgh and comparing this population's incidence of neoplasms compared to the general population, the former have a significantly elevated risk for developing neoplasm, which is 7.6 times higher for oropharyngeal cancer and 1.7 times higher for respiratory malignancies (Table 1).

Since a more prolonged exposure to immunosuppression is associated with an increased frequency of developing neoplasms, the cumulative risk of developing *de novo* malignancy rises from 20% at 10 years to 55% at 15 years after transplant[6]. In an Italian study analyzing 313 LT recipients who survived more than 12 mo after transplant, during a total follow-up time of 1753 person-years, *de novo* malignancies were diagnosed in 40 (12.8%) subjects, with a median time from transplantation to diagnosis of 54 mo (range, 2-159 mo)[7]. Other studies have reported a slightly lower mean interval between LT and diagnosis of non-lymphoid malignancies (36.2 mo, range, 5.8-74.1)[5].

Not only are malignant neoplasms more frequent in transplant recipients, but they also have a more aggressive behavior, present at an earlier age compared to the non-transplant population, and take a higher toll on survival[8]. Mortality after diagnosis of *de novo* malignant neoplasms is particularly elevated, with reported rates as high as 55% and a median survival of 54 mo after diagnosis[7]. Overall, estimated survival rates for all types of *de novo* malignancies are reportedly 70%, 56%, 48%, and 39% after 1, 3, 5, and 10 years, respectively. For certain types of cancer, mortality is particularly high, reaching 100% for lung cancer, 62.5% for esophageal and gastric cancers, 57% for head and neck cancer, 50% for post-transplant lymphoproliferative disorder (PTLD), and 50% for Kaposi Sarcoma (KS)[7].

Table 1 Estimated standardized incidence ratios for *de novo* malignancies after liver transplantation (data according to[7,9,15,46-48,61,72,174-182])

Cancer site/type	Estimated incidence (%)	SIR
All cancers	5-6	1.94-3
Kaposi's sarcoma	0.14-2.8	> 100
Skin (non melanoma)	0.9-3.2	> 30
PTLD	0.9-2.6	6-20
Gastrointestinal and oropharyngeal sites		
Lip/oropharyngeal/head and neck cancers	0.1-2.0	5-14
Esophagus[1]	0.5-1.19	12-18.7
Colorectal overall	0.0-0.65	1.41
Colorectal in IBD/PSC	0.7-7.9	3-5
Stomach	0.25	3
Vulva	0.25	8-23.8
Lung	0.6-1.2	2-8
Renal	0.35	2-2.65
Thyroid	0.20	4.60
Prostate	0.25-0.6	1 (risk not increased)
Breast	0.40	1 (risk not increased)
Colorectal in non-IBD/PSC	0.30	1 (risk not increased)

[1]Although there are no population-based SIR estimates showing an increased risk of esophageal cancer after LT, an Italian study reported an SIR of 23.4 on the basis of cases ascertained by medical record reviews[178]. This association may be related to prior alcohol exposure; 2 of 3 patients diagnosed with esophageal cancer in a US cohort underwent LT for ALD[1]. IBD: Inflammatory bowel disease; PSC: Primary sclerosing cholangitis; PTLD: Posttransplant lymphoproliferative disease; SIR: Standardized incidence ratio.

TYPES OF *DE NOVO* NEOPLASMS

De novo malignancies are neoplasms that develop after transplantation, including solid tumors such as pancreatic cancer, lung cancer, colorectal cancer, gastric cancer, esophageal cancer, renal cell carcinoma, bladder cancer, thyroid cancer, oral cancer, brain tumors and laryngeal cancer, as well as non-solid tumors, primarily PTLD/non-Hodgkin Lymphoma (NHL) and leukemia. According to a large German study analyzing the frequency and distribution of *de novo* neoplasms after LT[9], 1 *de novo* malignancy is to be expected approximately every 120 person-years after LT (120 *de novo* malignancies/14490 person-years). It was also shown that cancer incidence rates for LT

recipients are almost twice as high as those for an age- and sex-matched general population. To quantify the risk that the status of transplant recipient conveys, cancer site-specific incidence rates in the transplant population are compared against the general population, with standardized incidence ratios (SIRs). Estimated SIRs for each malignancy, as well as the reported incidence are shown in Table 1. PTLD is the most frequent *de novo* malignancy after LT, accounting for approximately 20% of cases[7]. Other common types of *de novo* malignant tumors include KS (17%), head and neck cancer (17%), esophageal tumors (12%), lung cancer (10%), gastric adenocarcinoma (7%), melanoma (5%), colorectal cancer (5%), cervical cancer (5%), and breast cancer (2%), as shown in a study from Northern Italy[7].

Skin cancer

In a series of LT recipients with nonlymphoid *de novo* malignancies, skin cancer was reportedly the most common type of malignancy (22/57 patients with *de novo* cancer, representing 33.3%), including squamous cell carcinomas in 50%, basal cell carcinomas in 40.9%, and melanomas in 9.1%. Neoplasms were most frequent on the skin of the head, face, and neck (in 14 subjects), but there were also several cases of multiple site involvement, and the mean time to onset was 36.4 mo (range, 8.2-75.1 mo)[5]. Another study demonstrated that the prevalence of pre-malignant and neoplastic cutaneous lesions increased with time, with a frequency of premalignant lesions of 5% at 2-3 years, 12% at 3-5 years, 28% beyond 5 years, and frequency of malignant lesions of 0% at 2-3 years, 9% at 3-5 years, and 12% beyond 5 years of follow-up after transplantation. Furthermore, in that same study, the cumulative incidence of cutaneous lesions was significantly higher in patients treated with cyclosporine compared to recipients on tacrolimus[10]. One-year survival after diagnosis of skin cancer in LT recipients is reportedly 90.9%[5]. Several factors have been identified as being considered high risk for developing skin cancer, including increased age, increased intensity and longer duration of immunosuppressive therapy, infection with human papillomavirus, history of increased ultraviolet exposure, easily burned skin, history of actinic keratosis, CD4 lymphocytopenia, and blue or hazel eyes[11,12]. Primary sclerosing cholangitis[13] as well as alcohol-related liver disease as indications for LT are associated with a higher risk of skin malignancies compared to other etiologies of liver disease[14,15]. Other risk factors for the development of skin malignancy after LT include male sex, age over 55 years, Caucasian background, and monoclonal antibody induction therapy[11], while the use of polyclonal or interleukin (IL)-2 receptor antibody induction therapy, treatment for rejection, and non-cholestatic etiologies of liver disease as indications for LT, seem not to be associated with an increased risk.

PTLDs

PTLD encompasses a heterogeneous group of diseases characterized by excessive proliferation of lymphoid cells and it commonly results from *de novo* infection or reactivation of latent Epstein-Barr virus (EBV)[16,17], especially in the case of EBV seronegative recipients of organs from EBV seropositive donors. LT carries an intermediate risk of PTLD, in contrast with intestinal transplantation, which has the highest rates[18,19]. An increased intensity of immunosuppression[5,20-23] and the use of certain types of immunosuppressive agents, in particular T-cell depleting antibodies such as Muromonab-CD3 (OKT3) or anti-thymocyte globulin (ATG), cyclosporine, and belatacept (in renal transplant recipients) constitute additional risk factors for PTLD development[24-26]. In an Italian study, 15 cases of PTLD were described in 1011 solid organ transplant recipients; in 13/15 patients, induction immunosuppressive therapy with OKT3 was used, and EBV was detected in 10 of 13 patients in whom neoplastic tissue was available for analysis. Moreover, in 2 of the 3 patients who were negative for EBV, hepatitis C virus (HCV) was present, and positivity for HCV was significantly more frequent in patients who developed PTLD compared to those who did not, suggesting a possible role of HCV in the development of PTLD[19]. Other studies have also shown a correlation between the presence of HCV and the development of PTLD[27-29].

In the pediatric population, PTLD is the most common tumor in solid organ recipients, with an overall incidence rate of 5% to 15% in different series or 298/100000 posttransplantation years of follow-up[30,31]. Reported mortality is unfortunately very high, of up to 60%, especially in infants who develop PTLD as a result of primary EBV transmission from EBV-positive allograft transplant[32-35].

The most important risk factors for PTLD development in the pediatric population include high levels of immunosuppression (especially associated with tacrolimus-based regimens[36]), young age, time from transplant (related to longer exposure time to immunosuppression), EBV seronegativity before transplant, and primary EBV transmission. Fukushima and collaborators, in a recently published study on 32 infants younger than 2 years who had undergone living-donor liver transplantation and were on tacrolimus-based immunosuppression, found that deteriorated tacrolimus metabolism (with elevated plasmatic levels) accompanied by an increase in Epstein-Barr viral load was more frequently associated with PTLD[36]. In a recently published paper by the Studies of Pediatric Transplantation Research Group[37] analyzing a large multicenter cohort of pediatric patients who underwent LT, transplants performed in the era 1995-2001 (*vs* those performed between 2002 and 2007), recipient EBV status, and frequent rejection episodes were associated with symptomatic EBV infection and

PTLD. The subgroup at a highest risk is constituted by younger infants with multiple rejection episodes. Importantly, the incidence of both symptomatic EBV infection and PTLD are seemingly decreasing in pediatric LT recipients, concomitantly with a reduction in immunosuppression[37].

In a recent study, Khedmat and Taheri[38] reviewed 250 cases of PTLD after liver transplantation published in the literature, of whom 212 were pediatric cases (18 years of age or less). PTLD was diagnosed at a mean age of 9.9 years and the mean ± SD interval between LT and diagnosis of PTLD was 28.7 mo (35.1 mo). Organs/areas involved included: orbit, skin, stomach, genitalia, central nervous system, spleen, kidneys, respiratory system, liver, bone marrow, small intestine, and colon; in comparison with their adult counterparts, histopathological features of PTLD were significantly of more benign types.

Analogous to management strategies in adults, a sequential approach is employed, starting with reduction or complete withdrawal of immunosuppression, initiation of inferferon-alpha, various chemotherapic regimens, surgery, and radiotherapy, escalating strategies if the previous alternative proves inefficacious[39]. Moreover, long-term withdrawal of immunosuppression has been shown to be feasible without graft rejection[40]. The use of the anti-B-cell monoclonal antibody rituximab has brought about improved results, and more recently, Gupta and collaborators reported on satisfactory outcomes employing a dual combination of rituximab and reduced dose chemotherapy, with two-year failure-free survival of 57% in liver transplant recipients[39].

Kaposi's sarcoma

KS is a multifocal angioproliferative mucocutaneous neoplasm driven by HHV-8 infection and represents approximately 4% of all post-transplant tumors. The risk of developing this neoplasm is increased 500-fold in solid organ transplant recipients compared with the general population[41,42]. In a large study on 2705 recipients of solid organs, amongst whom 159 LT recipients, KS was diagnosed in 1.44% of all transplant recipients, including 12.8% of LT recipients[43]. Contrary to most other neoplasms, the incidence of KS seems to decrease significantly with time after solid organ transplantation[44]. In the presence of infection with HHV-8, the most important risk factor for the development of this neoplasm is the intensity of immunosuppression, and its therapy is based on immunosuppression tapering, as well as the use of chemotherapeutic agents. Moreover, evidence is mounting on the usefulness of mTOR inhibitors in treating this tumor while at the same time providing effective immunosuppression[45].

Solid tumors

Lung cancer: The incidence of lung cancer among LT recipients is increased compared to the general population, and reportedly accounted for 15.7% of nonlymphoid neoplasms in a series of LT recipients, in whom it was diagnosed, on average, 48.5 mo (range, 11.2 to 64.3 mo) after LT, and a one-year survival of 37.5%[5]. In large case series of LT recipients, the mean time to diagnosis ranges from 42 to 50 mo[5,46-48]. Akin to the association between smoking observed in the general population, this carcinogen is correlated with an increased risk of lung cancer in transplant recipients[5,46]. Although probably representing an epidemiological association, as smokers are also frequently heavy drinkers, a study showed that patients with alcohol-related cirrhosis as an indication for LT had higher rates of lung cancer than those who underwent LT for other indications[49].

Head and neck cancers: Head and neck neoplasms are more frequent in the LT population than in the general population, and mean time to diagnosis is reportedly between 34.3 mo and 61.2 mo[5,15,47,50,51]. Oropharyngeal cancer is 25.5 times more frequent in patients transplanted for alcohol-related cirrhosis *vs* those transplanted for other indications[52]. Moreover, upper aerodigestive squamous carcinomas are more frequent in patients with alcohol-related cirrhosis as the main indication for LT[53]. Moreover, another study showed that whereas the incidence of oropharyngeal cancer was 16.7% in patients who underwent LT for alcohol-related liver disease, none of the patients who underwent LT for indications other than alcohol-related cirrhosis developed oropharyngeal malignant neoplasms (P = 0.001)[50]. Notably, there was not one case of oropharyngeal cancer in a small, single-center study involving patients without a history of smoking or alcohol use[54]. Likewise, tongue cancer and laryngeal cancer have been reported in smokers[5,46], and the carcinogenic effects of tobacco observed in the general population also applies for transplant recipients. It is difficult to establish the weight of alcohol compared to tobacco use as contributing risk factors for head and neck neoplasms, as alcohol is known to potentiate the carcinogenic effects of smoking[55], and also since patients who are heavy smokers also tend to be heavy drinkers[56].

Esophageal and gastric cancer: Although their incidence is increased with respect to the general population[57], gastric and esophageal cancers are reported infrequently in most series of LT recipients[58]. As well as for several other types of cancer, notably those of the oropharynx/larynx, alcohol is a well-established risk factor for esophageal malignant neoplasms[59], and this neoplasm occurs at a higher rate after LT in patients with alcohol-related liver disease[15,27,60]. In an Italian study on 313 LT recipients followed during a 15-year period, of 40 patients with *de novo* malignancy, esophageal cancer was diagnosed

in 12%, with a mortality (combined for esophageal and gastric cancer of 62.5%) being second only to that of lung cancer[7]. A German study analyzing 1,926 LT recipients found that 9 patients (0.5%) developed a *de novo* esophageal cancer and 1 patient developed cancer of the cardia (0.05%), diagnosed on average 51 mo after LT. The histological type of tumor was squamous cell carcinoma in 7/10 and adenocarcinoma in 3/10. Of note, 9/10 patients had undergone LT due to alcohol-related cirrhosis[61]. A predisposing lesion, Barrett's esophagus, has been demonstrated to rapidly evolve into adenocarcinoma after LT, which is why surveillance endoscopy with aggressive endoscopic treatment of Barrett's mucosa is paramount in these patients to prevent death from cancer[62-66]. In a Korean study of 6491 patients who underwent solid organ transplantation, 30 patients (0.46%) with 31 lesions were diagnosed with gastric cancer[67]. In another series, 36 cases of gastric cancer were identified among 7000 transplant-related malignant neoplasms, and 3 of the 34 were observed in LT recipients[68]. Moreover, another study reported 3 cases of gastric cancer amongst 329 cases of malignant neoplasms in LT recipients[69].

Genitourinary cancer: Although the incidence of prostate cancer does not seem to be increased in LT recipients, all other genitourinary cancers (including bladder and renal cancer) seem to be higher than that of the general population[5,15,27,46,47]. Mean time to diagnosis of non-prostate genitourinary cancer ranges from 20 to 55.3 mo, while in cases of prostate cancer the diagnosis is often performed between 5.8 and 18.4 mo after LT[5,15,47,48]. In LT recipients, prostate cancer is more often diagnosed at earlier stages and has a good prognosis, whereas renal and bladder cancers have a poor prognosis[5].

Gynecological cancer: Although it seems that breast cancer is no more frequent in LT compared to the general population[3], non-breast gynecological cancers (cervical and ovarian) are more frequent in LT recipients than in the general population[15,46,47]. It has been hypothesized that rigorous screening before LT has contributed to a tendency, albeit not statistically significant, for a lower incidence of breast cancer in LT recipients[5]. However, other studies have documented that breast cancer incidence is in fact elevated in the transplant population, with the advantage, however, that early detection is more common, and this has also resulted in decreased mortality compared to that of the general population upon similar diagnoses[46].

Colorectal cancer: The incidence of colorectal cancer seems to be higher in the LT recipient population *vs* the general population[46,47], although most of this difference in incidence, if not all, can be accounted for by the increased risk of colorectal cancer associated with LT for primary sclerosing cholangitis, probably due to the association with ulcerative colitis[70-72]. More frequently diagnosed between 16 and 50 mo after transplant, colorectal cancer in transplant recipients tends to be detected at an earlier age and has been associated with a worse prognosis compared to the general population[73,74].

***De novo* hepatocellular carcinoma:** A search performed by Trevisani *et al*[75] identified 14 cases of *de novo* hepatocellular carcinoma (HCC) which have been reported in the literature. Although until now a relatively rare occurrence, truly *de novo* HCC, that is, neoplasms arising from the liver graft and not recurrences of recipient HCC, might be seen more often in the future, due to the increased use of extended criteria grafts, especially those from older donors, donors carrying HCV or HBV infection, or alcoholic liver disease[76,77]. One of the principal risk factors for *de novo* HCC is recurrence of liver disease in the allograft, and especially the development of cirrhosis[75], and reported cases have been diagnosed on average 2 years after LT. As for non-transplant recipients, post transplant exposure to hepatocarcinogens like aflatoxin B1, nitrosamine, aromatic amines, vinyl chloride, azo-dyes, pesticides, arsenic, organic solvents, and cigarette smoking, can theoretically trigger the development of HCC, although no case has yet been reported in association with any of these factors. Immunosuppression regimens used in the 14 reported cases include OKT3, azathioprine, cyclosporine, corticosteroids, mycophenolate mofetil, basiliximab, and tacrolimus[78-82].

Prognosis seems dismal according to reported cases, despite tapering of immunosuppression, transarterial chemoembolization, radiofrequency ablation, hepatic resection, or retransplantation. Strategies for preventing this neoplasm include avoidance of recurrent graft damage as well as a judicious immunosuppression after LT[75]. While HCC recurrence is considered a contraindication for retransplantation, this therapeutic option could be contemplated in the setting of *de novo* HCC and has been reported in a case with development of this *de novo* malignancy 14 years after primary LT[82].

RISK FACTORS FOR THE DEVELOPMENT OF *DE NOVO* MALIGNANCIES

In a study analyzing risk factors for the development of solid neoplasms after LT, multivariate analysis demonstrated that primary sclerosing cholangitis (HR = 2.62, 95%CI: 1.50-4.56), alcohol-related cirrhosis (HR = 2.14, 95%CI: 1.22-3.73), smoking (HR = 1.72, 95%CI: 1.06-2.79), and increasing age in decades (HR = 1.33, 95%CI: 1.05-1.66) were all significantly associated with *de novo* neoplasms[1]. A summary of the most important risk factors is provided in Table 2.

Table 2 Risk factors for the development of *de novo* malignancies according to tumor location/type (data according to[5,14-17,20-22,25,26,46,48,50,53,54,61,62,64,75,130,181,183,184])

Tumor location/type	Risk factor
Skin	Age > 40 yr
	Male gender
	Skin type
	Sun exposure
	Smoking
	Alcoholic cirrhosis
	Primary sclerosing cholangitis as indication for LT
	Cyclosporine-based immunosuppression
KS	Increased intensity of immunosuppression
	Infection with HHV-8
PTLD	Age > 50 yr
	Infection with EBV (especially seronegative recipients of organs from EBV seropositive donors)
	Increased intensity of immunosuppression
	OKT3 or anti-thymocyte globulin
	Cyclosporine-based immunosuppression
	Hepatitis C virus
Lung cancer	Cigarette smoking
	LT for alcohol-related liver disease
Head and neck cancers	Cigarette smoking
	LT for alcohol-related liver disease
Esophageal and gastric cancers	LT for alcohol-related liver disease
	Barrett's Esophagus
Colorectal cancer	Primary sclerosing cholangitis
	Inflammatory bowel disease
De novo HCC	Recurrence of liver disease in the allograft
Gynecologic cancers	Insufficient evidence
Genitourinary cancers	Insufficient evidence

EBV: Epstein-Barr virus; HCC: Hepatocellular carcinoma; HHV-8: Human herpesvirus 8; KS: Kaposi's sarcoma; LT: Liver transplantation; PTLD: Post-transplant lymphoproliferative disorder.

DONOR-TRANSMITTED MALIGNANCIES

The role of immunosuppression in reactivating dormant neoplasms is supported by the fact that transplant recipients who have received organs from donors with previously cured neoplasms may develop the donor's malignancy[83,84]. Reportedly, 0.5% to 3% of donors have a history of malignancy, and transmission from these donors to the recipients has been demonstrated in 0.02%-6% of cases[85-89], the risk being higher in LT recipients as compared to recipients of other organs[90,91]. According to the time elapsed from clinical remission of the neoplasm in the donor to the moment of donation, tumor site, and risk of transmission, recommendations for specific tumor types have been issued by the Malignancy Subcommittee of the Disease Transmission Advisory Committee of the Organ Procurement and Transplantation Network/United Network for Organ Sharing (OPTN/UNOS). Organ shortage, a low risk of transmission of malignancy to the recipient, and the need for a life-saving transplant in cases of urgent LT may drive the decision of using organs from extended criteria donors, including donors with a neoplasm. It is important, however, to quantify the risk, based on the type of neoplasm. Thus, an organ from a donor with basal cell carcinoma is considered to be associated with a minimal risk (< 0.01%) of transmission and may be used as a graft, whereas at the other end of the spectrum, the history or presence of melanoma, lung cancer, or active breast cancer > stage 0 are considered at high risk of transmission (> 10%) and their use is discouraged[92]. Allegedly, organs from donors with central nervous system malignancies may be safely transplanted; in a study analyzing 62 recipients of organs from donors with a history of or active central nervous system neoplasm, 8 transmissions were identified, occurring 2-15 mo after transplant, with seven patients dying as the result of metastatic disease. The presence of one or more risk factors, identified as: high-grade tumors, ventriculoperitoneal or ventriculoatrial shungs, prior craniotomy and systemic chemiotherapy, entailed a risk of 53% of tumor transmission, whereas the rate was significantly lower (7%, $P < 0.01$) if no risk factor was present[93]. However, a more recent and larger study performed in the United Kingdom concluded that organs from donors who died as a consequence of primary intracranial malignancy, including those with high-grade tumors, should be considered for transplantation due to the small risk of tumor transmission. Identification of 448 recipients of 495 organs from 177 donors with primary intracranial malignancy, including 33 with high-grade malignancy (9 medulloblastomas and 24 grade Ⅳ gliomas amongst 179 donors) demonstrated not one single case of tumor transmission[94]. As in all medical interventions, a risk-benefit evaluation must be performed, the patient should be informed of the possibility of receiving one such organ, and this must be weighed against the risk of dying on the waiting list, which is much higher.

The recommendations for screening in the donor so as to reduce the risk of undiagnosed neoplasm and subsequent transmission to the recipient include execution of complete medical history specifically inquiring on previous diagnosis of malignancy, radiological imaging, complete physical examination to rule out possible skin cancer, laboratory analysis for the detection of tumor markers, pathology examination of extracted organs, and in cases of unexplained intracranial hemorrhage and in women with menstrual disorders, underlying neoplasms must be excluded[95,96].

IMMUNOSUPPRESSION

Immunosuppression plays a fundamental role in the development of neoplasms, acting through several different mechanisms including decreased immune surveillance, increased susceptibility to infections, induction of insulin resistance, and a direct carcinogenic effect which has been described in the case of some immunosuppressive agents. The association between

alterations in the immune system and the development of neoplasms is also reflected in the elevated incidence of cancer in most medical conditions associated with immunosuppression[97,98] and the fact that the length of exposure and intensity of immunosuppression correlate with the incidence of malignant neoplasms[99,100]. Whereas in immunocompetent subjects there is continuous ongoing surveillance that acts as tumor suppressor, keeping in check possible accumulated cell damage resulting in neoplasms, immunosuppression in organ transplant recipients results in a lower threshold for immunosurveillance, allowing neoplastic cells to proliferate.

Moreover, chronic immunosuppression renders transplant recipients more vulnerable to viral infections, some of which have oncogenic potential. Although not all neoplasms are the result of viral triggers, the ones that are tend to be those that show the greatest rise in frequency amongst transplant recipients including B-cell lymphoma and PTLD (EBV), squamous cell skin carcinoma (HPV), Kaposi's sarcoma (HHV8), anogenital cancers (HPV), Merkel skin cancer (polyomavirus), and HCC (HBV, HCV)[97]. The viral oncogenic potential may be enhanced by the action of some immunosuppressants. Calcineurin inhibitors in particular, can favor the expression of EBV growth and virus-inducing factors including IL-1, IL-6, and transforming growth factor (TGF-β), can promote EBV replication, and can augment immunoresistance by favoring the expression of anti-apoptotic genes[101].

Aside from these indirect effects, several immunosuppressive drugs seem to have direct oncogenic effects, either by provoking damage to DNA or through other mechanisms not linked to immunosuppression. Azathioprine, for instance, induces chromosomal aberrations and increases skin cell sensitivity to photodamage[97].

Calcineurin inhibitors: There is evidence of direct pro-oncogenic activity in the case of calcineurin inhibitors, which induce tumorigenesis and tumor growth by inducing cancer cell invasiveness[102], hampering DNA repair mechanisms[103,104] and apoptosis[103], inducing tumor angiogenesis *via* the stimulation of vascular endothelial growth factor (VEGF)[105], and promoting the transcription and functional expression of the TGF-β1 gene which results in tumor cell invasion and metastatic potential[106]. In LT recipients, it has been shown than exposure to elevated concentrations of tacrolimus (> 20 ng/mL) in the weeks immediately after transplantation increases long-term mortality due to infections, cardiovascular events and development of neoplasms[107-110].

Furthermore, both calcineurin inhibitors and steroids exert a diabetogenic effect, causing impaired insulin secretion and inducing pancreatic beta cell apoptosis[111-113]. As many as 5%-27% of LT recipients develop neo-onset diabetes mellitus, and it is associated with a negative impact on patient and graft survival[114-116], diabetes being a recognized risk factor for neoplasms, playing an important role especially in HCC[117]. Calcineurin inhibitors, especially tacrolimus, have in fact been shown to increase the risk of developing new-onset diabetes mellitus after transplantation.

Other immunosuppressant agents: The use of other immunosuppressant agents, including OKT3 and ATG, has also been associated with an increased risk for the development of neoplasms after solid organ transplantation. Early PTLD has been shown to occur shortly after administration of OKT3, with an average of 7 mo from transplantation and/or administration to diagnosis of PTLD[118]. In other series, high total doses of OKT3, especially in individuals in whom a second course of therapy was administered, were associated with a higher frequency of lymphomas[119,120]. In contrast, a single-center study reporting on 1570 LT of whom 125 patients developed *de novo* tumors, did not show any relationship between OKT3 and the development of *de novo* neoplasms; the authors note that this is consistent with the concept that chronic maintenance immunosuppression is more important than short albeit intense periods of immunosuppression (treated with OKT3)[47]. A recently published Cochrane Database Systematic Review evaluated the benefits and harms of immunosuppressive T-cell specific antibody induction compared with placebo, no induction, or another type of T-cell specific antibody induction for prevention of acute rejection in LT recipients, and included studies using T-cell specific antibodies polyclonal antibodies [rabbit of horse antithymocyte globulin (ATG), or antilymphocyte globulin (ALG)], monoclonal antibodies (OKT3, anti-CD2, or alemtuzumab), and IL-2 receptor antagonists (daclizumab, basiliximab, BT563, or Lo-Tact-1). The authors concluded that there were no statistically significant differences in terms of malignancy[121].

Mammalian target of rapamycin inhibitors: Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase downstream of the phophoinositide-3-kinase-related kinase family, which plays a fundamental role as regulator of various oncogenic processes including cell growth, proliferation, metabolism, and angiogenesis[122]. The combination of anti-tumoral as well as immunosuppressive properties render this family of drugs very attractive in the post-transplantation setting. There is growing evidence that the incidence of neoplastic disease is inferior in patients with gradual reduction of CNI with the introduction of mTOR inhibitors, *vs* those subjects treated with standard-dose CNI[123]. An anti-neoplastic activity has been demonstrated for everolimus with regard to various solid tumors, and a potential role in HCC and cholangiocarcinoma are being increasingly reported[105,124-127].

Table 3 Intensive screening protocols for tumor surveillance in liver transplant recipients (data according to[128-130])

Traditional screening	Intensive screening
Annual chest X-ray	Annual chest and abdominal CT
Annual abdominal ultrasound	Annual abdominal ultrasound
Chest and abdominal CT	Annual urologic screening with PSA determination
Mammography and urologic screening (with timing according to standard of care)	Annual Pap smear and mammography (every 1-2 yr)
	Annual skin examination
	Colonoscopy 1 year after LT in patients with adenoma on pre-LT colonoscopy, and repeated every 2-4 yr if more adenomas are found. Colonoscopy repetition every 10 yr in patients > 50-yr-old
	Ears, nose and throat clinic visit in patients with > 20 pack year smoking

CT: Computed tomography; PSA: Prostatic specific antigen; LT: Liver transplant.

PREVENTION

As most neoplasms are favored by immunosuppression, the long-term use of the lowest effective dose of immunosuppression to avoid rejection are recommended, as well as the avoidance of excessive sun exposure, treatment of premalignant lesions including warts and actinic keratoses, and avoidance of exposure to confirmed carcinogenic substances including those present in tobacco smoke.

Screening protocols are recommended in order to detect malignancies in early states, increasing the probability of opportune treatment and improving prognosis[128,129]. Some recommended strategies include monthly skin autoexam, annual dermatological visit, annual Pap smear, mammography every 2 years, annual digital rectal exam and prostate-specific antigen determination, annual fecal occult blood test, colonoscopy every 10 years, annual chest X-ray, abdominal ultrasound, chest and abdominal CT scan[130-135]. A summary of preventive measures is provided in Table 3.

Management of neoplastic disease in LT recipients: In general terms, management of malignant neoplasms in LT recipients is similar to that of the immunocompetent patient in terms of surgery, chemotherapy and radiotherapy, but, in contrast, one of the main pillars of the approach to a neoplasm in transplant recipients is represented by modification of immunosuppression, especially in tumors which are highly susceptible to immunosuppression, such as KS and PTLD.

Owing to their strong anti-angiogenic effects which result in inhibition of tumor growth, as well as their direct action on cancer cells by the inhibition of their dependence on the mTOR pathway for cell growth and survival, mTOR inhibitors are increasingly being used in the management of neoplasms in transplant recipients[105,136,137]. Since cyclosporine favors an invasive and aggressive tumor cell behavior, the combination with mTORi was hypothesized to be beneficial, adequately avoiding rejection while also providing malignancy control. This has been proven to be true, with significantly better survival times with mTORi plus cyclosporine treatment *vs* cyclosporine-only treatment in mice injected with tumor cells[138]. In fact, mTORi alone or mTORi plus cyclosporine impairs immunity and promotes allograft survival in experimental models, and the combination of sirolimus and everolimus with cyclosporine is effective in clinical transplantation, being approved by the Food and Drug Administration (FDA) for use in transplant recipients[139]. Specifically, everolimus is indicated for immunosuppression kidney heart and liver transplantation, while sirolimus has been approved for kidney transplantation. Malignancy rates post-conversion to sirolimus-based, CNI-free, immunosuppression regimen were significantly lower with respect to the CNI-based immunosuppression protocol in the RMR study and the CONVERT trials[140,141]. Moreover, experience in renal transplant recipients has demonstrated that the risk of *de novo* malignancies is significantly lower in patients treated with mTOR inhibitors (with or without CNIs) compared to patients on CNI-based regimens[142]. Thus, one of the recommended strategies in the management of post-transplant neoplasms is the conversion from CNIs to mTOR inhibitors or inclusion of mTOR inhibitors in a CNI-based immunosuppressive regimen[143-145]. Furthermore, in another study reporting on 10 LT recipients who had developed *de novo* neoplasms after LT, everolimus treatment significantly increased the probability of survival from 14% (in a similar historical cohort of patients not treated with everolimus) to 72% at 20 mo[146]. Moreover, in a recently published retrospective study analyzing prognostic factors for patients transplanted for alcohol-related cirrhosis who developed non-cutaneous *de novo* solid organ neoplasms, conversion to everolimus improved prognosis, with one- and five-year survival rates of 77.4% and 35.2% in patients converted to everolimus *vs* 47.2% and 19.4% in patients not treated with everolimus, respectively ($P = 0.003$)[147].

RECURRENCE OF NON-HEPATIC NEOPLASMS

With the broadening of eligibility criteria for LT, older patients are now being transplanted, increasing the probability of patients with past medical history of malignancy to be evaluated for LT, waitlisted, and transplanted. The risk of neoplastic recurrence upon commencement and maintenance of immunosuppression and its derived mortality must be weighed against the probability of survival without a transplant. Recurrence of a preexistent neoplasm can occur after

LT, and according to the risk of recurrence, neoplasms can be classified as low recurrence risk (0%-10%) as in the case of cervical carcinoma, endometrial carcinoma, myeloproliferative disorders, and lymphomas; intermediate recurrence rate (11%-25%) as in the case of colorectal cancer, non-melanoma skin cancer, and thyroid carcinoma; and neoplasms with a high recurrence rate (> 26%) as in the case of oral squamous carcinoma and breast cancer[148]. There is consensus that the tumor type and stage of the disease must be carefully evaluated, and according to this, recommendations have been made regarding the waiting time between achieving clinical "cure" or disease control and LT[149-151]. According to American[151] and European[95] guidelines, proposed malignancy-free delay periods before transplantation vary from no delay in cases of basal-cell skin cancers and incidental renal cell carcinoma, to less than 2 years in cases of small single focal neoplasms, low-grade bladder cancer, excised squamous cell carcinoma, 2 years in cases off testicular and thyroid neoplasms, to 2-5 years or more for malignant melanomas, breast cancer, invasive cervical cancer, and colorectal cancer. Nevertheless, since many patients being evaluated for LT are too sick to endure a long waiting period, provided that the neoplasm is adequately controlled and the stage of the neoplasm itself is not associated with a poor prognosis, LT may be considered before completion of the waiting period with informed consent of the candidate[152].

HCC RECURRENCE IN LT RECIPIENTS

In spite of the 5-year 60%-80% disease-free survival rate after LT for HCC in cases with unresectable early stages of the neoplasm, recurrence does occur in 3.5%-21% of cases, and is associated with a poor prognosis[153]. Tumor-related established risk factors for HCC recurrence after LT include high levels of alpha-fetoprotein[154,155], tumor grading[156,157], tumor stage[154,156-158], and vascular invasion[154,157,158], while immunosuppression-related risk factors for HCC recurrence are primarily the level of immunosuppression[156], mTOR- *vs* mTOR inhibitor-free immunosuppression regimen[154,159]. Clinical studies have shown a CNIs dose-dependent increase in the risk of developing HCC recurrence[102]. Elevated exposure to CNIs (mean trough concentrations of tacrolimus > 10 ng/mL or cyclosporine > 300 ng/mL) during the first postoperative period has in fact been associated with an increased risk of HCC recurrence[160]. Moreover, it has been observed that high doses of cyclosporine are associated with a lower recurrence-free survival in patients transplanted for HCC. In fact, a study on 219 patients transplanted for HCC undertaken in Milan revealed that elevated doses of cyclosporine or tacrolimus during the first 30 d after LT almost tripled the risk of HCC recurrence[127].

In contrast, mTOR inhibitors possess anti-angiogenic and anti-proliferative properties acting though the reduction of several growth factors and enhancing microvascular thrombosis, which correlates with lower metastatic potential[122,161]. The antineoplastic effect of mTOR inhibitors has also been shown in several clinical studies[162]. There is growing evidence that mTOR deregulation plays a significant role in hepatocellular carcinogenesis, and pre-clinical data indicate that deregulated expression of mTOR pathway effectors is present in 40%-50% of HCCs, and activation of the mTOR pathway is associated with less differentiated neoplasms, earlier tumor recurrence, and worse survival outcomes[163,164]. A recent meta-analysis comparing CNIs against sirolimus demonstrated a protective effect of the latter in terms of achieving a lower incidence of HCC recurrence after LT[165]. This protective effect was confirmed in a more recent meta-analysis[166], which demonstrated that sirolimus, compared with CNIs, was associated with lower HCC recurrence (OR = 0.30, 95%CI: 0.16-0.55, $P < 0.001$), lower HCC recurrence-related mortality (OR = 0.29, 95%CI: 0.12-0.70, $P = 0.005$), and lower overall mortality (OR = 0.35, 95%CI: 0.20-0.61, $P < 0.001$). In addition, a recent systematic review showed that patients on CNIs developed HCC recurrence significantly more frequently compared with patients on mTORi. In addition,patients on everolimus had significantly lower HCC recurrence rates compared with those on sirolimus or CNIs, although patients treated with mTOR inhibitors tended to have less advanced stages of HCC[167,168].

CONCLUSION

Overall, the risk of malignancy is two to four times higher in transplant recipients than in an age- and sex-matched population, and cancer is expected to surpass cardiovascular complications as the primary cause of death in transplanted patients within the next 2 decades[4,169]. *De novo* malignancy is a very significant cause of mortality, particularly for long-term survivors, and minimization of long-term immunosuppression should be aimed at reducing the incidence of *de novo* neoplasms[1,170]. Promising results in prevention of HCC recurrence have been reported with the use of mTOR inhibitors including everolimus and sirolimus[154,159,171] and the ongoing open-label prospective randomized controlled SILVER Study[172] will provide more information on whether sirolimus-containing *vs* mTOR-inhibitor-free immunosuppression is more efficacious in reducing HCC recurrence. The combined use of sorafenib, a multikinase antiangiogenic inhibitor, and an mTOR inhibitor has yielded positive results in treating patients with HCC recurrence after LT, despite notable associated toxity[173].

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TOPIC HIGHLIGHT

2015 Advances in Gastric Cancer

Clinical applications of 5-aminolevulinic acid-mediated fluorescence for gastric cancer

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Abstract

5-aminolevulinic acid (ALA) is a naturally occurring amino acid that is a protoporphyrin IX (PpIX) precursor and a next-generation photosensitive substance. After exogenous administration of ALA, PpIX specifically accumulates in cancer cells owing to the impaired metabolism of ALA to PpIX in mitochondria, which results in a red fluorescence following irradiation with blue light and the formation of singlet oxygen. Fluorescence navigation by photodynamic diagnosis (PDD) using ALA provides good visualization and detection of gastric cancer lesions and is a potentially valuable diagnostic tool for gastric cancer for evaluating both the surgical resection margins and extension of the lesion. Furthermore, PDD using ALA might be used to detect peritoneal metastases during preoperative staging laparoscopy, where it could provide useful information for the selection of a therapeutic approach. Another promising application for this modality is in the evaluation of lymph node metastases. Photodynamic therapy (PDT) using ALA to cause selective damage based on the accumulation of a photosensitizer in malignant tissue is expected to be a non-invasive endoscopic treatment for superficial early gastric cancer. ALA has the potential to be used not only as a diagnostic agent but also as a therapeutic drug, resulting in a new strategy for cancer diagnosis and therapy. Here, we review the current use of PDD and PDT in gastric cancer and evaluate its future potential beyond conventional modalities combined with a light energy upconverter, a light-emitting diode and near-infrared rays as light sources.

Key words: Fluorescence imaging; 5-aminolevulinic acid; Photodynamic diagnosis; Photodynamic therapy; Gastric cancer

Core tip: 5-Aminolevulinic acid (ALA) is a naturally occurring amino acid that is a protoporphyrin IX precursor and a next-generation photosensitive substance. Fluorescence navigation by photodynamic diagnosis (PDD) using ALA is a potentially valuable diagnostic tool for gastric cancer for evaluating both the surgical resection margins and the extension of the lesion. Furthermore, PDD using ALA might be useful to detect peritoneal metastases during preoperative staging laparoscopy and evaluation of lymph node metastases. Photodynamic therapy using ALA to cause selective damage based on the accumulation of a photosensitizer in malignant tissue is expected to become a non-invasive endoscopic treatment for superficial early gastric cancer.

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INTRODUCTION

5-Aminolevulinic acid (ALA) is a naturally occurring amino acid derivative that acts as an endogenous substrate and precursor to protoporphyrin IX (PpIX). PpIX is a heme precursor in the biosynthetic pathway that emits a strong red fluorescence upon excitation with blue light[1,2]. Since ALA is analogous to amino acids, rapid absorption can be expected following ingestion, followed by being immediately metabolized to heme in normal cells. In various cancer cells, exogenous administration of excessive amounts of ALA increases the cellular level of PpIX, resulting in a higher accumulation of PpIX in cancer cells than in normal cells. However the mechanism of preferential accumulation of PpIX remains unclear[3-5].

Gastric cancer remains one of the leading causes of cancer-related deaths and is the third most common cancer worldwide[6]. Although surgery is the main treatment for operable gastric cancer, most patients who present with inoperable advanced or metastatic disease require palliative treatment, including chemotherapy or radiotherapy, in combination with novel molecular-targeted drugs that induce antibody-dependent cellular cytotoxicity[7-9]. Fortunately, early gastric cancer (EGC), in which the cancer cells are confined to the gastric mucosa or submucosa, regardless of lymph node metastasis, has an excellent outcome with surgical curative resection[10,11]. A less invasive therapy using endoscopy, such as endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD), is considered a favorable treatment for EGC without lymph node metastasis because it is able to resect a target lesion *en bloc* with preservation of the entire stomach[12,13].

Recent advances in limited treatments, including EMR, ESD and minimally invasive surgery, can improve the quality of life for patients with EGC[11,14]. However, sufficient resection margins are necessary to prevent the reappearance of EGC, as inadequate resections that do not maintain surgical margins free of cancer can lead to disease recurrence[10,11]. Recently, fluorescence imaging using photosensitive molecules such as ALA or indocyanine green (ICG) has been developed, and it is being applied as a navigating tool for various fields of surgery[15,16].

PRINCIPLE OF PHOTODYNAMIC DIAGNOSIS

Exogenously administered ALA is incorporated by cells and is used to synthesize a naturally fluorescent substance, PpIX, which also exhibits photoactivity. When PpIX is excited by irradiation of a specific wavelength, mainly visible blue light of 375-475 nm, it emits red fluorescence, and this property can be harnessed to accurately identify cancer cells, which accumulate PpIX. This so-called photodynamic diagnosis (PDD) is a relatively new modality that is based on tumor-specific accumulation of 5-ALA-induced PpIX[3,15,16].

PDD imaging systems were recently improved to enable detection of malignant lesions in the brain, lung and esophagus based on systemic administration of the photosensitive substance Photofrin[15]. However, Photofrin has considerable adverse effects, such as strong phototoxic skin reactions and increases in serum aminotransaminase. Accordingly, ALA is clinically recognized as an effective and safe substrate for detecting various cancers owing to the low risk of side effects [3,15,17-19].

We have used an endoscopic PDD system (Karl Storz, Tuttlingen, Germany) comprised of a CCU Tricam SLII/3CCD CH Tricam-P PDD, D-Light C, and HOPKINSII Straight Forward Telescope 30° (Karl Storz)[5,15,16,20]. The D-Light C light source (300 W xenon arc lamp, Karl Storz) is equipped with a band-pass filter designed to transmit blue light (excitation wavelength, 375-445 nm), and the CCU Tricam SLII/3CCD CHTricam-P PDD video camera system is equipped with a long-pass filter designed to exclude blue light for fluorescence imaging (fluorescence emission wavelength, 600-740 nm). This PDD system has the advantage that it can switch instantly between the blue light mode for fluorescence imaging and the white light mode for conventional observation. In our studies, ALA is dissolved in 50 mL of a 5% glucose solution, and 1.0 g of this solution is given orally 3-4 h before the intraoperative PDD observation. Patients are shielded from direct sunlight for 24 h to avoid phototoxicity. In our experience, no special precautions have been necessary during ALA-PDD, such as liver

Table 1 Previous clinical reports of 5-aminolevulinic acid-mediated fluorescence for gastric cancer

Study	Year	Number of patients	Clinical application	Results
Mayinger *et al*[22]	1999	4	Feasibility study of PDD during endoscopy	All malignant lesions exhibited fluorescence during PDD
Kishi *et al*[20]	2012	13	Detection of peritoneal metastases	The tumor detection rate was higher in PDD than white light (72% *vs* 39%)
Murayama *et al*[21]	2012	13	Detection of peritoneal metastases	The accuracy of the fluorescence imaging was greater than that of white-light imaging
Koizumi *et al*[23]	2013	14	Detection of metastatic lymph nodes	The sensitivity, specificity, and accuracy of ALA-PDD were 70.8%, 96.7%, and 92.4%, respectively
Namikawa *et al*[16]	2014	21	Feasibility study of PDD during surgery	The sensitivity, specificity, and accuracy of ALA-PDD were 57.7%, 100%, and 66.7%, respectively
Nakamura *et al*[24]	2014	5	Evaluation of high-resolution magnifying videoendoscopy for PDD and PDT	PDD and PDT were successfully and safely performed, and CR was obtained in 71.4% of cases
Kishi *et al*[25]	2014	52	Detection of peritoneal metastases	ALA-PDD detected peritoneal metastases in 21% of the patients, while 46% of the patients had no evidence of dissemination on white-light examination

ALA: 5-aminolevulinic acid; CR: Complete response; PDD: Photodynamic diagnosis; PDT: Photodynamic therapy.

support or light shielding, and no adverse events have thus far been encountered.

PDD FOR GASTRIC CANCER

Several studies have used PDD using ALA (ALA-PDD) for the diagnosis and treatment of gastric cancer, including the application of this approach for staging laparoscopy[16,20-22]. Table 1 summarizes previous clinical reports of ALA-mediated fluorescence used for gastric cancer, including both PDD and photodynamic therapy (PDT)[16,20-25]. Among these studies, we recently examined the clinical usefulness of ALA-PDD during surgery for gastric cancer[16]. Our findings indicate that there is a difference in the ALA-PDD-positive rate between intestinal- and diffuse-type gastric cancers.

Oligopeptide transporters (PEPT), such as peptide transporter 1 (PEPT1) and PEPT2, are involved in the cellular uptake of ALA[26-29]. ALA-mediated PpIX accumulation in tumors is associated with the expression of particular proteins, such as PEPT1, PEPT2, ferrochelatase, and ATP-binding cassette transporter G2 (ABCG2)[29-31]. Hagiya *et al*[29] reported that high expression of PEPT1 and low expression of ABCG2 correlated with ALA-induced accumulation of PpIX. Furthermore, Kobuchi *et al*[30] reported that ALA-induced PpIX production and cellular photosensitivity correlated negatively with the expression of the PpIX transporter ABCG2 but not with that of PEPT1, PEPT2 or ferrochelatase in a cancer cell line. These differences in the PpIX biosynthesis pathway might involve diagnostic findings of different histological types of gastric cancer[29,30,32].

Regarding the future direction of ALA-PDD for gastric cancer, it could be used as a tool for evaluating surgical resection margins and thereby assist with pathological diagnosis during surgery. Sometimes, a situation is encountered in which decisions must be made for proceeding during surgery for the treatment of gastric cancer, such as determining the extent of the cancer in cases with indistinct margins[33]. In these cases, ALA-PDD might provide useful information to judge the margins that would be sufficient for resection of the tumor.

Magnifying endoscopy is useful for determining the extent of intramucosal spread of differentiated EGC, and also provides more precise endoscopic diagnoses[34,35]. In addition, ALA-PDD might be an effective diagnostic procedure during endoscopic therapies such as EMR or ESD, in addition to early detection of gastric cancer. To detect any extension of the cancer in these limited surgeries[36], new endoscopic diagnostic procedures for EGC based on magnifying endoscopes and image-enhanced endoscopies, such as narrow-band imaging (NBI) and flexible spectral imaging color enhancement (FICE), have been developed[34,35,37]. In this regard, ALA-PDD could provide additional information to that obtained with these conventional endoscopic modes, and thus might prove valuable for evaluating the extent of the lesion.

NEW APPROACH FOR CANCER STAGING

Peritoneal metastasis from a primary gastric cancer is the most frequent type of distant metastasis and post-surgical recurrence in advanced gastric cancer with serosa-invading tumors, and is an incurable condition with poor prognosis[38,39]. Numerous attempts with new approaches have been made for gastric cancer staging, because accurate staging of gastric cancer, and particularly the diagnosis of peritoneal dissemination, is a prerequisite for determining the most appropriate therapy[7,8,25,39]. Although staging laparoscopy is used frequently in the management of patients with advanced gastric cancer to prevent unnecessary laparotomy[40,41], it has limitations in visualizing the dissemination of cancer nest[42-45].

Staging laparoscopy with ALA-PDD is safe and

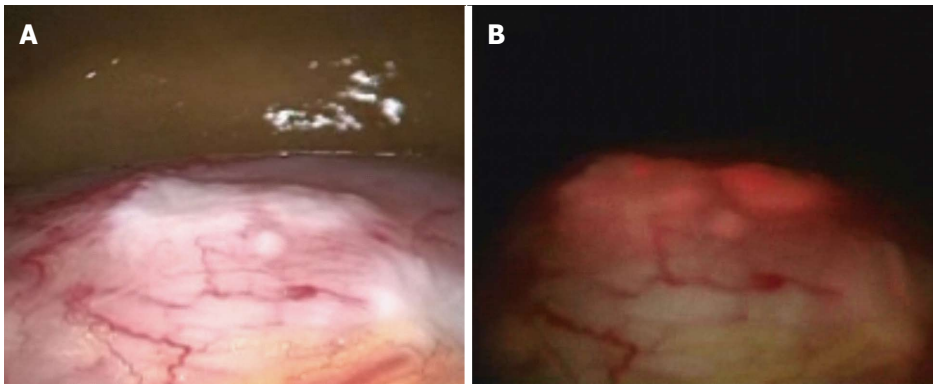

Figure 1 Staging laparoscopy images using photodynamic diagnosis-5-aminolevulinic acid showing serosal invasion of gastric cancer. A whitish nodulated surface was seen in the stomach (A), which was visualized by ALA-induced red fluorescence (B). ALA: 5-aminolevulinic acid.

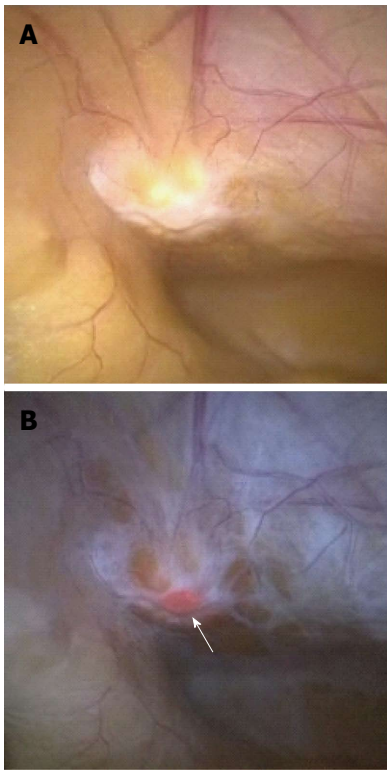

Figure 2 Peritoneal metastasis of gastric cancer comparing photodynamic diagnosis-5-aminolevulinic acid with conventional methods during staging laparoscopy. ALA-induced red fluorescence was seen in the white nodule (B, arrow), which appeared as a non-specific nodule under white light (A). ALA: 5-aminolevulinic acid.

improves the diagnostic accuracy for peritoneal metastases in patients with gastric cancer[20,21,25]. Kishi *et al*[20,25] examined the usefulness of ALA-PDD with staging laparoscopy in patients with serosa-invading advanced gastric cancer, comparing the detection sensitivity with that obtained using conventional white light. They demonstrated that the tumor detection rate of 72% using ALA-induced fluorescence was significantly higher than that achieved using white light (39%) in a mouse model of peritoneal metastases, which involved 8 mice with 729 peritoneal nodes. In addition, three metastatic lesions that were invisible under white light were detected under ALA-induced fluorescence in 13 patients undergoing staging laparoscopy. Furthermore, they correlated the ALA-PDD results with those from peritoneal fluid cytology and molecular diagnostic testing in 52 patients with advanced gastric cancer[26]. Twenty-four of the 52 patients (46%) had no macroscopic evidence of peritoneal metastases on white light examination; however, ALA-PDD detected dissemination in 5 of these 24 patients (21%).

Murayama *et al*[21] assessed the diagnostic capability of fluorescence laparoscopy in 13 patients with advanced gastric cancer using ALA for peritoneal dissemination, and for small superficial liver metastases that are difficult to identify by computed tomography scanning. Five of the 13 patients demonstrated peritoneal metastases, and one patient demonstrated superficial liver micrometastases by fluorescence laparoscopy using ALA.

We have also carried out staging laparoscopy using ALA-PDD in patients with serosa-invading gastric cancer and confirmed good visualization of peritoneal metastases (Figures 1-3). Thus, ALA-PDD in combination with staging laparoscopy is a promising procedure for improving the reliability of detection of invisible metastases in advanced gastric cancer.

Lymph node metastases are one of the most important prognostic factors in gastric cancer, and precise diagnosis of these metastases is essential for selecting the most appropriate therapeutic strategy. Koizumi *et al*[23] examined the feasibility of using ALA-PDD to detect metastatic foci in 144 excised lymph nodes that were obtained from 14 gastric cancer patients. In total, 121 lymph nodes were diagnosed in agreement with the results of histopathological examination, and the overall diagnostic accuracy of gross fluorescence inspection without regard to fluorescence patterns was 84%. The authors concluded that ALA-PDD is a feasible and direct approach for detecting metastatic lymph nodes in gastric cancer patients using a simple apparatus. One difficulty with ALA-PDD in this application is that fluorescence imaging

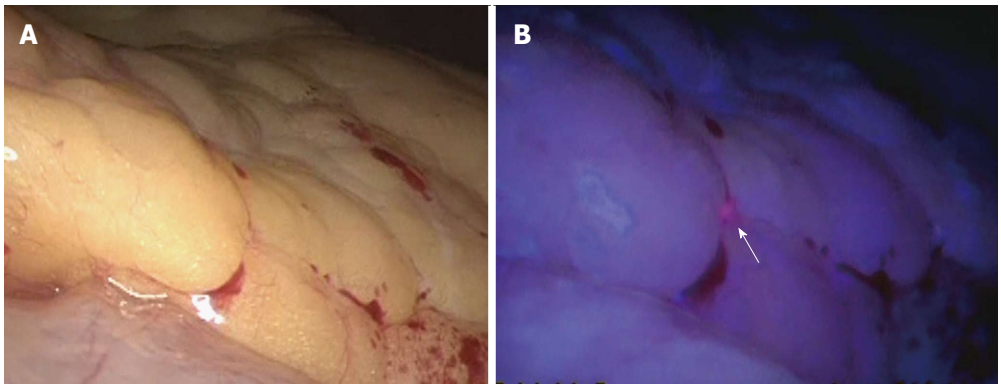

Figure 3 Peritoneal metastasis of gastric cancer detected by photodynamic diagnosis using 5-aminolevulinic acid. ALA-induced red fluorescence was seen in the omentum (B, arrow), which was not detected under white light (A). ALA: 5-aminolevulinic acid.

is only obtained from the outside of the lymph nodes, because human lymph nodes are surrounded by abundant connective tissues, causing a lower depth of penetration of blue light into the tissues. If deep observations were possible, diagnosis of lymph node metastasis would be possible in the full surgical field *in situ*, and thus it could be possible to select individual lymph nodes for extraction[21]. To resolve these issues, further investigations are needed.

PHOTODYNAMIC THERAPY FOR GASTRIC CANCER

ALA has been used successfully, not only to diagnose, but also to treat various tumors[44,45]. PDT is defined as the use of photodynamic agents that are biochemically activated by light, to cause tissue damage in the treatment of disease[46]. During PDT, emission of the excitation light, which falls within the absorption wavelengths of PpIX, results in the generation of reactive oxygen species (ROS) that induce apoptosis within the irradiated cells[47-49]. One advantage of ALA-PDT is that the lack of PpIX in normal cells potentially allows for a tumor-specific PDT with minimal adverse effects, and because tissue penetration by the excitation light is limited, the cytotoxic effects of PDT are also limited to the superficial tissue[50]. Therefore, ALA-PDT is expected to become an important non-invasive endoscopic treatment for superficial EGC[50,51].

Loh *et al*[46] demonstrated selective photosensitization of gastric mucosa with sparing of the other tissue layers of the stomach with ALA-PDT using a rat model. Although red light at a peak wavelength of approximately 635 nm is often used as the excitation light source in ALA-PDT[52,53], Hino *et al*[50] reported the efficacy of a light-emitting diode (LED) as an irradiation source for ALA-PDT in a mouse model of peritoneally disseminated gastric cancer. They demonstrated differences in anticancer effects, including ROS generation and cytotoxic effects, among three LED sources, which were violet at a peak wavelength of 410 nm, green at a peak wavelength of 525 nm, and red at a peak wavelength of 635 nm. The violet and green LEDs had the same anticancer effects, which were significantly greater than those of the red LED[50]. Thus, violet light has a greater cytotoxic effect, while red and infrared light penetrate deeper within biological tissues for *in vivo* clinical applications[51].

The near-infrared (NIR) window, composed of wavelengths between 700 and 1000 nm, provides the greatest depth of penetration in biological tissues, while blue or red lights yield low tissue penetrability and therefore are limited to surface cancer applications. To this end, Shimoyama *et al*[51] demonstrated a potentially novel PDT in a human gastric cancer cell line using NIR-irradiated lanthanide nanoparticles (LNP), which are excited by NIR-emitting visible light and administered using ALA. In addition to the tissue penetrability advantage, these early studies indicated possible additional advantages of LNP-sensitizer conjugates in reducing the background fluorescence, photobleaching, and photoblinking properties that are generally associated with such techniques[51,54].

CONCLUSION

ALA-PDD is a promising and safe diagnostic modality for determining tumor extent and for detecting metastatic lesions in gastric cancer. Furthermore, PDT has potential advantages in terms of minimizing the procedure invasiveness. Further investigations, including a prospective randomized controlled trial, are needed to verify the usefulness of the ALA-mediated fluorescence technology for gastric cancer.

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TOPIC HIGHLIGHT

2015 Advances in Inflammatory Bowel Disease

Advances in refractory ulcerative colitis treatment: A new therapeutic target, Annexin A2

Satoshi Tanida, Tsutomu Mizoshita, Keiji Ozeki, Takahito Katano, Hiromi Kataoka, Takeshi Kamiya, Takashi Joh

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Abstract

Medical treatment has progressed significantly over the past decade towards achieving and maintaining clinical remission in patients with refractory ulcerative colitis (UC). Proposed mediators of inflammation in UC include pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-2, and the cell-surface adhesive molecule integrin α4β7. Conventional therapeutics for active UC include 5-aminosalicylic acid, corticosteroids and purine analogues (azathioprine and 6-mercaptopurine). Patients who fail to respond to conventional therapy are treated with agents such as the calicineurin inhibitors cyclosporine and tacrolimus, the TNF-α inhibitors infliximab or adalimumab, or a neutralizing antibody (vedolizumab) directed against integrin α4β7. These therapeutic agents are of benefit for patients with refractory UC, but are not universally effective. Our recent research on TNF-α shedding demonstrated that inhibition of annexin (ANX) A2 may be a new therapeutic strategy for the prevention of TNF-α shedding during inflammatory bowel disease (IBD) inflammation. In this review, we provide an overview of therapeutic treatments that are effective and currently available for UC patients, as well as some that are likely to be available in the near future. We also propose the potential of ANX A2 as a new molecular target for IBD treatment.

Key words: Tumor necrosis factor-α; Shedding; Integrin α4β7; Epidermal growth factors

Core tip: The main goal of ulcerative colitis (UC) therapy is to induce and maintain long-term corticosteroid-free remission. Therapies such as anti-tumor necrosis factor (TNF)-α and integrin α4β7 neutralizing antibodies

have emerged in recent times, but are not universally efficacious; additional treatments are needed. We have recently demonstrated that annexin (ANX) A2 inhibition may be a new therapeutic strategy to prevent TNF-α shedding during inflammatory bowel disease (IBD) inflammation. Here we focus on effective therapies for UC patients that are currently available, or will be in the near future, and the potential of ANX A2 as a new molecular target for IBD treatment.

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INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory disease that affects the colonic mucosa and that is characterized by repeated periods of remission and deterioration[1]. Pharmacologic management of UC to achieve clinical remission, and improve the quality of life currently consists of 5-aminosalicylic acids (5-ASA)[2], corticosteroids[3], purine analogues [azathioprine (AZA) and 6-mercaptopurine (6-MP)][4], cytapheresis[5] [granulocyte and monocyte adsorptive apheresis (GMA) and leukocytapheresis (LCAP)], calcineurin inhibitors[6,7] [cyclosporine and tacrolimus (TAC)], and biologics including tumor necrosis factor (TNF)-α inhibitors[8,9]. In particular, anti-TNF-α antibodies such as infliximab (IFX)[8] and adalimumab (ADA)[9] can induce clinical remission in patients with refractory UC by inhibiting the activity of TNF-α, a member of the TNF superfamily that mediates a series of immune responses. However, responses to anti-TNF-α antibodies are often diminished during scheduled maintenance therapy; consequently, patients develop flare-ups[10]. Therefore, new therapeutic targets are needed for UC patients who no longer respond to these therapeutic agents.

A disintegrin and metalloproteinase (ADAM)17, also known as TNF-α converting enzyme (TACE)[11,12], is a key enzyme for the shedding of the membrane-anchored TNF-α (proTNF-α). We have recently demonstrated that annexin (ANX) A2 is involved in the shedding of proTNF-α through ADAM17[13]. Inhibition of ANX A2 may be a new therapeutic strategy for the prevention of TNF-α shedding during inflammatory bowel disease (IBD) inflammation.

The present review focuses on therapeutic treatments that are effective and currently available for UC patients, or will be in the near future, and the potential of ANX A2 as a new molecular target for IBD treatment.

5-ASA

A systematic review and meta-analysis of the effect of 5-ASA on UC demonstrated that 5-ASA is highly effective for inducing remission in UC with a relative risk (RR) of failure to achieve remission of 0.79 (95%CI: 0.73-0.85; P = 0.009). This finding was based on analysis of data showing that remission of UC was not achieved in 887 (60.3%) of 1470 patients randomized to receive 5-ASA, compared with 494 (80.2%) of 616 patients allocated to placebo[14]. In addition, when remission was defined as endoscopic healing[15-19], 5-ASA was of benefit in inducing remission in active UC (RR = 0.76; 95%CI: 0.69-0.84). Moreover, a systematic review and meta-analysis that investigated the effect of high- or standard-dose 5-ASA (≥ 2 g) *vs* low-dose 5-ASA (< 2 g) on induction of remission demonstrated that doses of ≥ 2 g/d were more effective than doses of < 2 g/d for inducing remission with a RR of failure to achieve remission of 0.91 (95%CI: 0.85-0.98)[14]. This finding was based on data showing that 380 (58.7%) of 647 patients receiving high- or standard-dose 5-ASA failed to achieve remission, compared with 257 (69.8%) of 368 patients assigned to low-dose 5-ASA[18,20-26].

A systematic review and meta-analysis of the efficacy of 5-ASA *vs* placebo in preventing relapse in quiescent UC demonstrated that 5-ASA is highly effective for preventing relapse in UC with a RR of relapse of 0.65 (95%CI: 0.55-0.76)[14]. This finding was based on data showing that 342 (40.3%) of 849 patients randomized to 5-ASA relapsed, compared with 409 (62.6%) of 653 patients allocated to placebo[27-37].

It was also suggested that doses of ≥ 2 g/d may be more effective than doses of < 2 g/d for preventing relapse with a RR of relapse of 0.79 (95%CI: 0.64-0.97). This finding was based on data showing that 225 (34.7%) of 649 patients receiving high- or standard-dose 5-ASA relapsed, compared with 379 (42.8%) of 885 patients assigned to low-dose 5-ASA[14].

Corticosteroids

A systematic review and meta-analysis of the efficacy of corticosteroids in UC demonstrated that standard corticosteroids were superior to placebo for UC remission with a RR of failure to achieve remission of 0.65 (95%CI: 0.45-0.93)[38]. This finding was based on analysis of data showing that 122 (54.0%) of 226 patients assigned to standard oral glucocorticoids failed to achieve remission, compared with 173 (79.0%) of 219 patients allocated to placebo[3,39-42]. Based on the above, standard corticosteroids are probably effective in inducing remission in UC.

This systematic review also showed that there was no evidence of increased adverse events in patients taking standard corticosteroids, compared with placebo, even though the absolute rate was higher (14.3% compared with 7.0%, RR = 1.69; 95%CI:

0.30-9.62)[38].

Cytapheresis

Cytapheresis including GMA (Adacolumn®) and LCAP (Cellsoba®) is an extracorporeal therapy that selectively depletes activated granulocytes and monocytes, or leukocytes, resulting in amelioration of the gut inflammation of UC.

A systematic review and meta-analysis of the effect of GMA in both active and corticosteroid-dependent or resistant UC demonstrated that GMA appeared superior to conventional medical therapy. This conclusion was based on data showing that 26 (74%) of 35 patients assigned to GMA achieved remission, compared with 16 (49%) of 35 patients receiving prednisolone (PSL) (P = 0.02)[43,44]. In addition, there was also evidence for corticosteroid-sparing effects with GMA, with significantly lower cumulative doses of corticosteroids, and significantly higher rates of corticosteroid-free remission in patients receiving GMA. These findings were based on data that showed that (1) during the 12 wk of treatment, the cumulative amount of PSL received per patient was 1157 mg in 46 patients assigned to GMA, compared with 1938 mg in 23 patients assigned to receiving the mean dose of PSL up to 30 mg daily (P = 0.001)[45]; and that (2) 27 (77%) of the GMA-treated patients achieved corticosteroid-free at 12 wk, compared with 5 (14%) of the patients allocated to PSL (P = 0.008)[43]. However, GMA did not achieve significantly higher remission rates compared with a sham procedure in achieving remission in UC[46]. Interestingly further subgroup analysis demonstrated that GMA is of benefit in patients with confirmed endoscopically active disease. This conclusion was based on the data of a total of 63 patients with histological evidence of mucosal erosions or ulcerations at baseline, which showed that clinical remission was achieved in 11 (24%) of 46 patients randomized to GMA, compared with 0 (0%) of 17 patients allocated to sham apheresis (P = 0.03). On the other hand, a randomized trial comparing LCAP with a sham column also suggested benefit[47]. Interestingly, a systematic review and meta-analysis of the effect of intensive GMA regimens (two sessions per week) over conventional GMA regimens (one session per week) in achieving remission in UC demonstrated that intensive GMA regimens had higher remission rates[48-50] and shorter time-to-remission than conventional regimens[48,49]. Serious adverse side effects have been rare in patients receiving GMA. Based on the above, cytapheresis appears of some benefit in UC.

AZA/6-MP

The traditional pyramid of therapy for the management of UC suggests that patients are prescribed immunosuppressive agents when 5-ASA and corticosteroids fail[51]. A systematic review and meta-analysis of the effect of the immunosuppressant AZA on active UC demonstrated a trend to benefit of AZA over placebo in a total of 130 UC patients allocated to AZA or placebo, with no statistical significance (RR = 0.85; 95%CI: 0.71-1.01; P = 0.07)[4,52,53]. However, AZA is of benefit in preventing relapse in quiescent UC (RR = 0.60; 95%CI: 0.37-0.95; P = 0.03)[52]. This finding was based on data that 26 (39.3%) of 66 patients receiving AZA experienced a relapse of UC, compared with 40 (65.6%) of 61 patients allocated to placebo[4,53,54], with a statistically significant benefit of AZA.

Based on the above, AZA/6-MP appears to be of little benefit for inducing remission in active UC, but may prevent relapse in quiescent UC.

This systematic review also showed that there was no evidence of increased adverse events in patients taking purine analogues, compared with placebo[52]. However, there has been one trial that reported that one patient was dying of an infection associated with an immunocompromised state that occurred when taking AZA[55]. AZA/6-MP are also associated with a 4-6 fold increased risk of lymphoma[56,57] and a 2-6 fold increase in non-melanoma skin cancer[58,59]. Thus, immunosuppressive therapy with AZA/6-MP is never without risk.

Calcineurin inhibitors

Calcineurin inhibitors including cyclosporine and TAC are useful for the treatment of refractory UC due to their potent immunosuppressive properties that inhibit the transcription of the early activation genes encoding interleukin (IL)-2, TNF-α, and interferon-γ, which contribute to the development of inflammation[60].

A clinical trial that investigated the effect of cyclosporine on severely active UC, in which a response was defined as symptomatic improvement demonstrated that cyclosporine was of benefit over placebo in improving symptoms (RR no improvement with cyclosporine, 0.22; 95%CI: 0.07-0.67). This finding was based on data showing that 2 (18%) of 11 patients receiving cyclosporine had no response, as compared with 9 of 9 patients allocated to placebo[7,52].

A recent systematic review of pertinent literature in the Cochrane Database that investigated the efficacy of TAC in inducing remission or clinical improvement of symptoms of UC in a total of 63 moderate-to-severe UC patients randomized to TAC or placebo demonstrated that TAC was of benefit in inducing short-term clinical improvement in patients with refractory UC. This conclusion was based on data showing that 21 (48.8%) of 43 patients randomized to TAC achieved clinical improvement at 2 wk, compared with 2 (10.0%) of 20 patients allocated to placebo (odds ratio (OR), 8.66; 95%CI: 1.79-42.00), with a statistically significant benefit of TAC[60,61]. However, TAC is of little benefit in inducing remission. This conclusion is based on data showing that 6 (13.9%) of 43 patients randomized to TAC achieved remission at 2 wk, compared with 1 (5.0%) of 20 patients allocated

to placebo (OR = 2.27; 95%CI: 0.35-14.75), with no statistically significant benefit of TAC over placebo.

Regarding safety concerns, patients in the high serum target concentration group were significantly more likely than placebo patients to experience adverse events related to treatment (P = 0.043). Finger tremor (n = 6) was the most common adverse event in 43 patients receiving TAC. Other adverse events included: gastroenteritis, sepsis, sleepiness, hot flush, headache, queasiness and stomach discomfort[60,61].

Based on the above, TAC may be effective for short-term clinical improvement in patients with refractory UC.

Biological therapy

IBDs are characterized by chronic inflammation involving the surplus or excessive activity of the immune system in the gut. In order to block this excessive immune reaction, many approaches to the treatment of IBD with biological agents against inflammatory cytokines and adhesive molecules have been developed. The most popular approach to IBD treatment is to block TNF-α, a pro-inflammatory cytokine, which activates inflammatory cells, up-regulates adhesion molecules, and ultimately induces gut inflammation. Treatments with monoclonal antibodies against TNF-α are currently successful in many patients. However, only a third or less will achieve remission and many of those who do will eventually lose their response[62,63]. Monoclonal antibodies (vedolizumab) that block integrin α4β7, which mediates the infiltration of leukocytes into the gut mucosa, have also been developed, and will hopefully be used in clinical practice in the near future.

A very recent systematic review and network meta-analysis of the efficacy of biological agents on UC in a total of 2282 mild-to-moderate UC patients randomized to biological agents (n = 1167) or placebo (n = 1115) also demonstrated that all biological agents (ADA, golimumab (anti-TNF-α), IFX, and vedolizumab) were superior to placebo for induction of clinical response, clinical remission, and mucosal healing, except for ADA for clinical remission. Furthermore, IFX was shown to be more likely to induce a favorable clinical outcome than ADA for induction of clinical response (OR = 2.36, 95%CI: 1.22-4.63), clinical remission (OR = 2.79, 95%CI: 0.95-8.83), and mucosal healing (OR = 2.02, 95%CI: 1.13-3.59)[8,9,64-68]. In addition, all biological agents also suggested superiority over placebo for maintenance[64].

TNF-α BLOCKADE

Another systematic review and meta-analysis of the efficacy of all anti-TNF-α antibodies on moderately to severely active UC demonstrated that IFX antibodies are superior to placebo in inducing remission (RR of failure to achieve remission, 0.72; 95%CI: 0.57-0.91). This conclusion is based on data showing that remission of UC was not achieved in 231 (42.9%) of 539 patients that were randomized to receive IFX for 6 to 12 wk, compared with 201 (69.8%) of 288 patients allocated to placebo[8,69-72].

Regarding safety concerns, it was also suggested that the number of patients experiencing any adverse event was not greater with IFX in moderate-to-severe UC[69]. Based on the above, IFX is of benefit over placebo in inducing remission in active UC.

IFX and calcineurin inhibitors such as cyclosporin and TAC are effective for the treatment of patients with moderate or severe corticosteroid-dependent/refractory UC. Whether cyclosporin or TAC therapy should precede IFX as a second-line therapy currently remains controversial. A parallel, open-label randomized controlled trial compared the efficacy of cyclosporin and IFX on acute severe UC that was refractory to intravenous corticosteroids. In this trial, a total of 115 severe UC patients were randomized to cyclosporine (n = 58) or IFX (n = 57), and this trial demonstrated that cyclosporine was not more effective than IFX. This conclusion was based on data showing that 35 (60%) of 58 patients receiving cyclosporine failed to respond to the treatment by day 98, compared with 31 (54%) of 57 patients receiving IFX, with no statistically significant benefit of cyclosporine over IFX (OR = 1.3; 95%CI: 0.6-2.7; P = 0.52). Furthermore, 50 (86%) of 58 patients receiving cyclosporine achieved a clinical response by day 7, compared with 48 (84%) of 57 patients receiving IFX, with no statistically significant benefit of cyclosporine over IFX (OR = 1.2; 95%CI: 0.4-3.3; P = 0.76)[73].

A retrospective study that investigated the efficacy of IFX salvage therapy for patients with severe or moderate UC who failed to respond to TAC demonstrated that IFX salvage therapy following TAC tended to be more efficacious in TAC responders (loss of response or no tolerance) than in non-responders (refractoriness), and that sequential therapy may prove useful and well tolerated. These conclusions were based on data showing the following: (1) in 13 patients receiving IFX for severe or moderate UC who showed refractoriness or loss of response to TAC, or no tolerance, the mean partial Mayo score of UC activity was significantly decreased (P < 0.05) to 5.69, 3.07, and 2.77 at baseline, 8, and 30 wk, respectively; (2) six (46.2%) of the 13 patients showed clinical remission at 8 wk and four (30.8%) showed clinical remission at 30 wk; and (3) rates of clinical remission at 8 and 30 wk of IFX therapy were 60.0% and 40.0%, respectively in TAC responders, and good remission rates of 37.5% and 25.0%, respectively, were also obtained in TAC non-responders[74]. More interestingly, some recent investigations demonstrated that biological therapy could be terminated after achieving complete remission in response to scheduled maintenance therapy with TNF-α biologics in patients

with refractory UC[75,76].

Combination therapy with IFX plus AZA

A randomized, double-blind, double-dummy trial evaluated the efficacy of IFX alone, AZA alone, and combination therapy with IFX and AZA, at week 16 for the treatment of moderate-to-severe UC that was naïve to anti-TNF-α antibodies in a total of 239 patients. This trial demonstrated that the patients receiving combination therapy with IFX and AZA were more likely to achieve corticosteroid-free remission at week 16 than those receiving either monotherapy. This conclusion was based on data showing that 31 (39.7%) of 78 patients receiving a combination of IFX and AZA achieved corticosteroid-free remission, compared with 17 (22.1%) of 77 patients receiving IFX alone (P = 0.017) and 18 (23.7%) of 76 patients receiving AZA alone (P = 0.032)[77]. In addition, combination therapy led to significantly better mucosal healing than AZA alone, based on data showing that 49 (62.8%) of 78 patients receiving IFX and AZA combination therapy achieved mucosal healing at week 16, compared with 42 (54.6%) of 77 patients receiving IFX alone (P = 0.295) and 28 (36.8%) of 76 patients receiving AZA alone (P = 0.001)[77].

Vedolizumab

A recent systematic review of pertinent literature in the Cochrane Database that investigated the efficacy of vedolizumab for induction and maintenance of remission in a total of 606 moderate-to-severe UC patients randomized to vedolizumab demonstrated that vedolizumab is significantly more effective than placebo in inducing clinical remission and response as well as endoscopic remission. This conclusion was based on the following data: (1) 293 (77%) of 382 patients that were randomized to vedolizumab failed to achieve clinical remission by week 4 to 6, compared with 205 (92%) of 224 patients allocated to placebo, with a statistically significant effect in favor of vedolizumab (RR = 0.86; 95%CI: 0.80-0.91); (2) 48% of patients randomized to vedolizumab failed to have a clinical response at week 6, compared with 72% of patients allocated to placebo (RR = 0.68; 95%CI: 0.59-0.78); and (3) 68% of patients failed to achieve endoscopic remission at week 4 to 6, compared with 81% of patients allocated to placebo (RR = 0.82; 95%CI: 0.75-0.91)[78]. In addition, vedolizumab was of benefit over placebo in preventing relapse in patients who were in remission, based on data that showed that 140 (54%) of 247 patients randomized to vedolizumab experienced a clinical relapse at week 52, compared with 106 (84%) of 126 patients allocated to placebo (RR = 0.67; 95%CI: 0.59-0.77)[78]. Regarding safety concerns, patients receiving vedolizumab were no more likely than those receiving placebo to experience adverse events or serious adverse events[78].

Based on the above, vedolizumab is superior to placebo for induction of clinical remission, response, and endoscopic remission in patients with moderate-to-severe UC, and for prevention of relapse in patients with quiescent UC.

Tofacitinib

Tofacitinib is an inhibitor of Janus kinases 1, 2 and 3 that are believed to block lymphocyte activation, function, and proliferation through inhibition of signaling involving gamma chain-containing cytokines including IL-2, -4, -7, -9, and -15[79,80]. Consequently tofacitinib is expected to be a therapeutic agent for the treatment of active UC.

A double-blind, placebo-controlled, phase II trial that evaluated the efficacy of tofacitinib in 194 patients with moderate-to-severe UC demonstrated that clinical response at week 8 occurred in 20 (42%) of 48 patients allocated to placebo (95%CI: 28-56) compared with 10 (32%) of 31 patients randomized to 0.5 mg of tofacitinib (95%CI: 16-49; P = 0.39), 16 (48%) of 33 randomized to 3 mg of tofacitinib (95%CI: 31-66; P = 0.55), 20 (61%) of 33 randomized to 10 mg of tofacitinib (95%CI: 44-77; P = 0.10), 38 (78%) of 49 randomized to 15 mg of tofacitinib (95%CI: 66-89; P < 0.001)[81]. In addition, clinical remission at week 8 occurred in 5 (10%) of 48 patients receiving placebo (95%CI: 2-19), compared with 4 (13%) of 31patients receiving 0.5 mg of tofacitinib (95%CI: 1-25; P = 0.76), 11 (33%) of 33 receiving 3 mg of tofacitinib (95%CI: 17-49; P = 0.01), 16 (48%) of 33 receiving 10 mg of tofacitinib (95%CI: 31-66; P < 0.001), and 20 (41%) of 49 receiving 15 mg of tofacitinib (95%CI: 27-55; P < 0.001)[81]. Regarding safety concerns, there was a dose-dependent increase in both low-density lipoprotein and high-density lipoprotein cholesterol concentrations at week 8 with tofacitinib, which reversed after discontinuation of the study drug.

Based on the above, patients with moderate-to-severe UC treated with tofacitinib were more likely to achieve clinical response and remission than those receiving placebo.

ANX A2 as a new molecular target for conquering the failure of TNF-α blockade

As described above, TNF-α blockade using anti-TNF-α antibodies is not always successful. A better understanding of the TNF-α shedding process may lead to new methods of blocking TNF-α shedding and thereby attenuating the inflammation of UC. A recent investigation demonstrated a mechanism for the regulation of TNF-α shedding in which ANX A2 regulates ADAM17-mediated cleavage and subsequent shedding of proTNF-α from the cell membranes of monocytes and colon epithelial cells[13].

ANX A2 was initially isolated as a substrate for the tyrosine kinase of the oncogene protein pp60

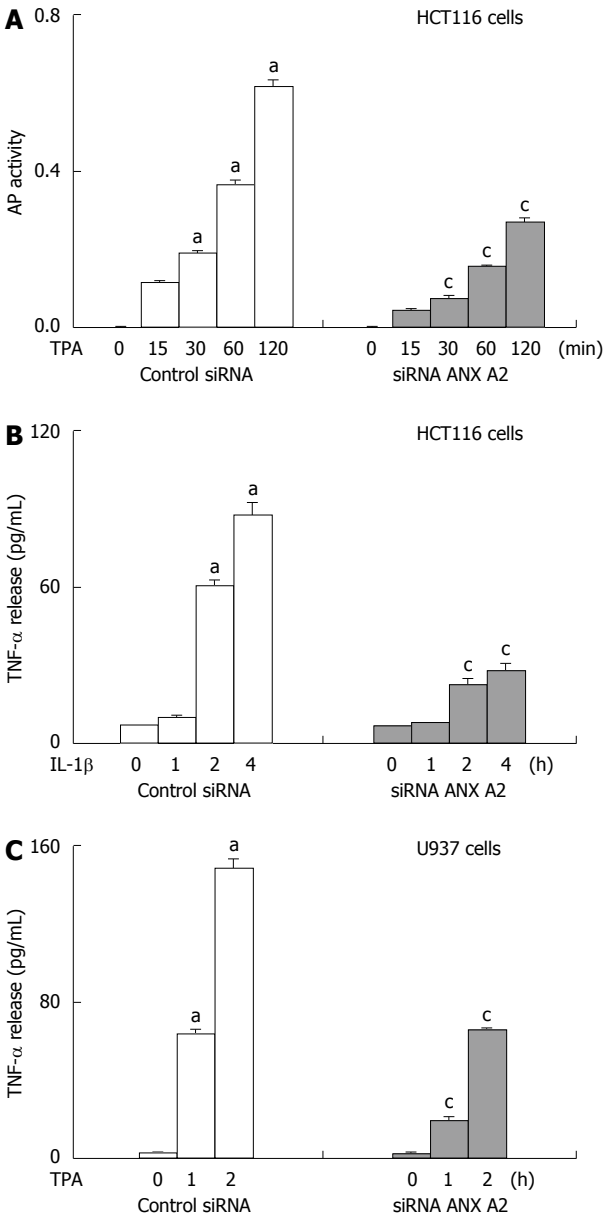

Figure 1 Involvement of annexin A2 in tumor necrosis factor-α ectodomain shedding and release. Quoted and adapted from Ford *et al*[14]. A: Quantitative analysis of TNF-α shedding during TPA stimulation of HCT116 cells overexpressing AP-tagged proTNF-α and inhibitory effects of ANX A2 siRNA on TNF-α shedding. $^{a}P < 0.05$ *vs* control for the stimulatory effect, and $^{c}P < 0.05$ *vs* control for the inhibitory effect; B: Inhibitory effect of ANX A2 siRNA on IL-1β-induced TNF-α release from HCT116 cells. The concentration of TNF-α released into the conditioned medium was measured using ELISA. $^{a}P < 0.05$ *vs* control for the stimulatory effect, and $^{c}P < 0.05$ *vs* control for the inhibitory effect; C: Inhibitory effects of ANX A2 siRNA on TPA-induced TNF-α release in U937 cells. The concentration of TNF-α released into the conditioned media was measured using ELISA. $^{a}P < 0.05$ *vs* control for the stimulatory effect, and $^{c}P < 0.05$ *vs* control for the inhibitory effect. TNF: Tumor necrosis factor; ANX: Annexin; IL: Interleukin.

(v-src)[82]. ANX A2 is a pleiotropic calcium- and anionic phospholipid-binding protein that exists as a monomer and as a heterotetrameric complex with the plasminogen receptor protein, S100A10[83]. A recent extensive study of the detailed biological functions of ANX A2 showed that ANX A2 in complex with S100A10 participates in Ca^{2+}-evoked exocytosis, and, in the endocytic pathway, ANX A2 in combination with acylated caveolin is considered to be involved in the internalization/transport of lipids[84]. Therefore, ANX A2 has been proposed to play a key role in many processes including exocytosis, endocytosis, membrane organization, ion channel conductance, and in linking the F-actin cytoskeleton to the plasma membrane[83,85].

Interaction of ANX A2 with ADAM 17 is required for TNF-α ectodomain shedding

The molecular mechanism by which TNF-α shedding is induced by interaction of ANX A2 with ADAM 17 has now become clear. TNF-α is known to be expressed on the cell membranes of monocytes. Western blotting that examined the endogenous protein expression levels of TNF-α, ADAM17 and ANX A2 in cell lines such as the colon epithelial cell lines, HCT116 and HT29, and the monocyte cell line, U937, showed that a high level of TNF-α protein was constitutively expressed in U937 cells, whereas HCT116 and HT29 cells expressed TNF-α at very low levels. High expression levels of ADAM17 and ANX A2 were observed in all three cell lines. Immunoprecipitaion with an anti-ANX A2 antibody followed by Western blotting with an anti-ADAM17 antibody demonstrated that ADAM17 directly interacts with ANX A2. It is known that the ectodomain of proTNF-α is mainly shed through the activity of ADAM17, although ADAM10 can also mediate a small amount of proTNF-α shedding[86]. The role of ADAM17 in TNF-α shedding was confirmed by analysis of the inhibitory effects of KB-R7785, an ADAM inhibitor, and of ADAM17 short interfering RNAs (siRNAs) on 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced shedding of TNF-α from HCT116 cells overexpressing alkaline-phosphatase (AP)-tagged proTNF-α, which allowed quantitative analyses of shed AP-tagged TNF-α in the culture medium during TPA stimulation using an AP assay. Forced depletion of ANX A2 using siRNAs targeted towards ANX A2 resulted in a significant suppression of TPA-induced TNF-α shedding (Figure 1A). In accordance with these data, the expression level of the AP-tagged proTNF-α protein of these HCT116 cells was decreased after TPA stimulation, which was partially inhibited by siRNA-mediated depletion of ANX A2. Furthermore, forced depletion of ANX A2 using siRNAs targeted toward ANX A2 resulted in a significant suppression of stimulation-induced endogenous TNF-α release from HCT116 and U937 cells, which was assessed using an ELISA of TNF-α shed into the conditioned medium (Figure 1B and C). These data suggested that ANX A2 is involved in TNF-α shedding and release in colon epithelial cells and monocytes.

ANX A2 depletion promotes ectodomain shedding of epidermal growth factor receptor ligands

ADAM17 is also a key enzyme for the shedding of

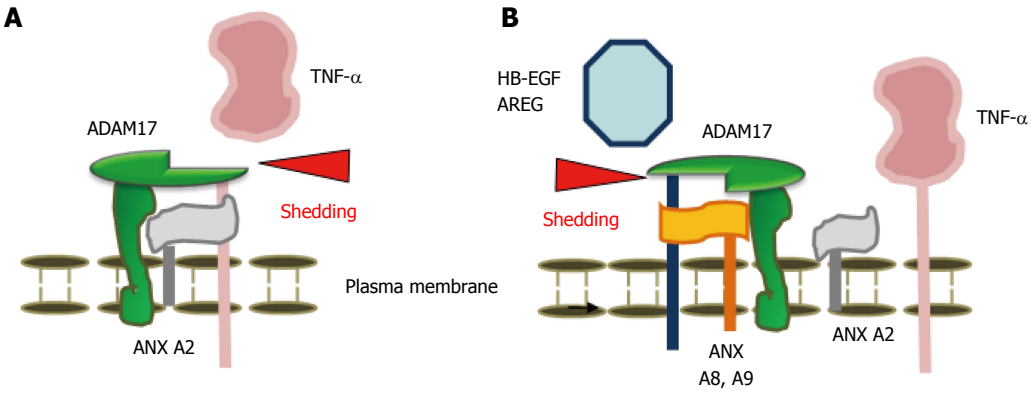

Figure 2 Putative model of ADAM17-cleaved shedding of tumor necrosis factor-α or epidermal growth factor receptor ligands through annexins. Quoted and adapted from Ford *et al*[14]. A: Highly expressed ANX A2 (grey) binds to and activates ADAM17 (green) and, as a consequence, ANX A2 promotes ADAM 17-mediated ectodomain shedding of TNF-α (pink). In parallel, the levels of ANX A8 and 9 are regulated so that they are maintained at a low level; B: Highly expressed ANX A8 and A9 (orange) subsequently bind to and activate ADAM17; as a consequence, ANX A8 and A9 promote ADAM 17-mediated ectodomain shedding of EGFR ligands. In parallel, levels of ANX A2 are regulated so that low levels are maintained. TNF: Tumor necrosis factor; ANX: Annexin; IL: Interleukin; EGFR: Epidermal growth factor receptor.

various other membrane proteins in addition to TNF-α, including epidermal growth factor receptor (EGFR) ligands[87]. The C-terminus of type 1 membrane proteins such as EGFR ligands [*e.g.*, amphiregulin (AREG) and heparin-binding epidermal growth factor-like growth factor (HB-EGF)] is in the cytoplasm, whereas the N-terminus of type 2 membrane proteins (*e.g.*, TNF-α) is in the cytoplasm. The detailed mechanism by which ADAM17 cleaves type 1 and 2 membrane proteins is unclear. An AP assay of HCT116 cells overexpressing AP-tagged proAREG and proHB-EGF demonstrated that, in contrast to its effect on TNF-α shedding, depletion of ANX A2 with siRNAs significantly increased AREG and HB-EGF shedding[13]. These experiments confirmed that ANX A2 is involved in the ectodomain shedding of AREG and HB-EGF.

The combined data indicated that depletion of ANX A2 inhibited ADAM17-mediated ectodomain shedding of proTNF-α; conversely, depletion of ANX A2 upregulated ADAM17-mediated ectodomain shedding of proAREG and proHB-EGF. These results suggest that depletion of ANX A2 ameliorates gut inflammation by suppressing TNF-α cleavage and induces cell proliferation and mucosal repair by promoting AREG and HB-EGF cleavage.

More interestingly, depletion of other members of the ANX family, ANX A8 and A9 abrogated the shedding of EGFR ligands, suggesting that ANX A8 and A9 are required for their shedding[88]. In contrast, decreased levels of TNF-α shedding were observed during stimulation from HCT116 cells overexpressing ANX A8 and A9, compared with mock cells, suggesting that ANX A8 and A9 inhibit TNF-α shedding.

Based on the above studies, ANX A2, A8 and A9 are responsible for regulation of the cleavage of the type 2 membrane-anchored protein TNF-α, and the cleavage of the type 1 membrane-anchored proteins AREG and HB-EGF (Figure 2). Clearly, ANX A2 is a new candidate molecular target for overcoming the failure of TNF-α blockade, and inhibition of ANX A2 may be a new therapeutic strategy for the prevention of TNF-α shedding during IBD inflammation.

CONCLUSION

As detailed in this review, many agents with different mechanisms of action are available, or are likely to be available in the near future, for the treatment of UC. However, these therapeutic strategies are not always satisfactory and there also remains the problem of refractory UC. Of the current treatments, calcineurin inhibitors, TNF-α blockade, and vedolizumab, which block or neutralize the production and functions of proinflammatory cytokines including IL-2 and TNF-α, and adhesive molecules, can be effective for the treatment of patients with refractory UC, but they have limitations. To address these limitations, the development and clinical trials of new therapeutic agents that target the surplus or excessive activity of the immune system are needed. ANX A2, which mediates TNF-α shedding, is also one such new candidate molecular target. Progress in understanding the pathogenesis of UC is expected to result in the emergence of many potentially useful treatments, such as the targeting of ANX A2, for UC treatment in the future.

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REVIEW

Role of the normal gut microbiota

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Abstract

Relation between the gut microbiota and human health is being increasingly recognised. It is now well established that a healthy gut flora is largely responsible for overall health of the host. The normal human gut microbiota comprises of two major phyla, namely Bacteroidetes and Firmicutes. Though the gut microbiota in an infant appears haphazard, it starts resembling the adult flora by the age of 3 years. Nevertheless, there exist temporal and spatial variations in the microbial distribution from esophagus to the rectum all along the individual's life span. Developments in genome sequencing technologies and bioinformatics have now enabled scientists to study these microorganisms and their function and microbe-host interactions in an elaborate manner both in health and disease. The normal gut microbiota imparts specific function in host nutrient metabolism, xenobiotic and drug metabolism, maintenance of structural integrity of the gut mucosal barrier, immunomodulation, and protection against pathogens. Several factors play a role in shaping the normal gut microbiota. They include (1) the mode of delivery (vaginal or caesarean); (2) diet during infancy (breast milk or formula feeds) and adulthood (vegan based or meat based); and (3) use of antibiotics or antibiotic like molecules that are derived from the environment or the gut commensal community. A major concern of antibiotic use is the long-term alteration of the normal healthy gut microbiota and horizontal transfer of resistance genes that could result in reservoir of organisms with a multidrug resistant gene pool.

Key words: Normal gut microbiota; Bioinformatics; Health; Immunomodulation; Metabolic function

Core tip: In this review we present an up-to-date overview of the normal gut microbiota, their functional implications in health, and the mechanistic insights that orchestrate these functions. We also discuss the characteristics that define a healthy gut microbiota and factors that shape and perturb the gut microbial diversity and functions. The evidence that we present here is a composite of observational and experimental studies on humans, germ free and humanized mice.

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INTRODUCTION

Microbiota refers to the entire population of micro-organisms that colonizes a particular location; and includes not just bacteria, but also other microbes such as fungi, archaea, viruses, and protozoans[1]. Significant interest have evolved on the gut microbiota in the recent years within the scientific community; and the gut microbiota have been associated with a large array of human diseases ranging from luminal diseases such as inflammatory bowel diseases (IBD)[2] and irritable bowel syndrome (IBS)[3], metabolic diseases such obesity and diabetes[4], allergic disease[5] to neurodevelopmental illnesses, though the strength of evidence is not robust with many of them. It has been speculated since long that the gut microbiota bear significant functional role in maintaining the gut in the normal individual and human health as a whole. There is now mounting evidence resulting from studies on humans and germ free mice that supports these speculations. Several high quality data from the US Human Microbiome Project (HMP)[6], European Metagenomics of the Human Intestinal Tract (MetaHIT)[7] and several other studies have now demonstrated the beneficial functions of the normal gut flora on health down to the genetic level. For example, studies have now identified several gut microbial genes, such as the HMO-related gene cluster 1 that is responsible for human milk oligosaccharide digestion.

From an immunological perspective, micro-organisms are viewed as pathogens by the host immune system that recognizes and eliminates them. However, majority of the gut bacteria are non-pathogenic and, co-habit with the enterocytes in a symbiotic relationship. The gut commensals predominantly aid in nutrient metabolism, drug metabolism, prevention of colonization of pathogenic microorganisms and in intestinal barrier function. At the same time, the immune system has co-evolved to live in a collaborative relationship with the healthy microbiota, while serving its function to fight off invasive pathogenic microorganisms.

The purpose of this manuscript is to review the recent evidence on the functions of the normal gut microbiota and the mechanistic insights into the execution of these pro-health functions. Data presented in this review is a composite of both observational and experimental studies on humans, germ free and humanized mice. The implications of the gut microbiota in disease states are out of the scope of this review.

CURRENT METHODS TO STUDY GUT MICROBIOTA

To study the gut microbiota, stool samples have to be collected from individuals and DNA from stool is isolated. Isolation, identification and enumeration of the vast majority of gastrointestinal microorganisms using conventional culture based techniques is an arduous task. Earlier, using culture based techniques, scientists were able to isolate only 10%-25% of the microbiota, and this was because most of the microorganisms in the gut are anaerobic. Later, with the improvements in the anaerobic culturing techniques, dominant genera were identified such as, *Bacteroides, Clostridium, Bifidobacterium etc*. The major drawback in using these techniques is the difficulty in studying the culture characteristics of various colonies on a petri plate. Secondly, it is time consuming[8-10].

With the availability of high throughput gene sequencing technology, study of the gut microbiota currently consists of two major stages: (1) 16S rRNA based sequencing of bacterial gene; and (2) bioinformatics analysis. Metabolomics is another rapidly expanding field of gut microbiota research that evaluates small molecules associated with the interrelationship of host-bacterial metabolism that has implications in health and disease. Composite data from the gut microbiota and the metabolome currently provides the most powerful evidence that can demonstrate the closest association with health and diseased states.

Bacterial gene sequencing

Sequencing of bacterial genes involves metagenomic analysis of DNA that codes for the 16S rRNA. The 16S region of bacterial gene is small (1.5 Kb size) and highly conserved, with 9 hyper variable sites that are sufficient to differentiate various bacterial species[11]. Common regions for bacterial identification in 16S rRNA are the V3, V4, V6 and V8[12]. With the development of biomedical technology, bacterial gene sequencing has rapidly evolved from Sanger's sequencing to several variations of next-generation sequencing (NGS). Even though NGS could provide

Table 1 Advantages and disadvantages of few of the currently available next generation sequencing techniques[15,16]

Techniques used in next generation sequencing	Accuracy	Advantages	Disadvantages
454 Pyrosequencing	99.9%	Less amount of sample, long read lengths, large number of samples can be easily read	Homopolymer errors Expensive
Shot gun Sequencing	98%	Short reads in short time	Assembly process is computationally expensive
Illumina Sequencing (Sequencing by synthesis)	98%	Accurate, quicker, reliable and cheap	Expensive
Pacific Bio Sequencing (single molecule real-time sequencing)	99.9%	Fast and provides long read length	Expensive equipment
Ion Torrent Sequencing (Ion semiconductor)	98%	Fast and less expensive equipment	Multiple monomer errors
SOLiD (Sequencing by Ligation) Sequencing	99.9%	Less expensive when compared to other methods	Slow and difficult to sequence palindromes

voluminous data with fair to good accuracy, they are not free from problems. A recent study have shown that sequencing could be prone to errors that most likely results from the library preparation methods and choice of primers[13]. The other issue of concern in 16S rRNA based sequencing is the variability of results across different sequencing centers, both for predominant and minor taxa. This variation again could be result of differences in primers used to generate the amplicon libraries[14]. Table 1 presents the accuracy, advantages and disadvantages of the currently available sequencing techniques[15,16].

BIOINFORMATICS ANALYSIS

The data obtained from sequencing is often voluminous, fragmented, noisy, overlapping, and contaminated. Bioinformatics analysis enables cleaning up the data and the identification of the bacterial taxa. This can also be extended to obtaining information also on metabolic functions using a wide array of bioinformatics platforms. Furthermore, statistical analysis of the sequence data also help in identifying alpha diversity (diversity of species within the same individual), beta diversity (inter-individual species diversity), relative abundance, and several other parameters related to the organisms. Figure 1 shows the workflow of study of the gut microbiota.

COMPOSITION OF THE NORMAL GUT MICROBIOTA

Even though it was earlier thought that the gut microbiota comprised of 500-1000 species of microbes[17] a recent large scale study has estimated that the collective human gut microflora is composed of over 35000 bacterial species[18]. Furthermore, if defined from a perspective of total bacterial genes, the Human Microbiome Project and the Metagenome of the Human Intestinal tract (MetaHIT) studies suggest that there could be over 10 million non-redundant genes in the human microbiome. A Danish study of the gut microbiome and their function involving 123 non-obese and 169 obese individuals resulted in the concept of high gene count (HGC) and low gene count (LGC), both of which have implications in health and disease[19]. The HGC microbiome includes *Anaerotruncus colihominis, Butyrivibrio crossotus, Akkermansia* sp., *and Fecalibacterium* sp.; with a high *Akkermansia* (Verrucomicrobia): *Ruminococcus torque/gnavus* ratio. The defining features of HGC microbiome in favour of a digestive health includes increased proportion of butyrate producing organisms, increased propensity for hydrogen production, development of a methanogenic/acetogenic ecosystem and reduced production of hydrogen sulfide[19]. The HGC individuals have a functionally much robust gut microbiome and lower prevalence of metabolic disorders and obesity. On the other hand, LGC individuals harbor a higher proportion of pro-inflammatory bacteria such as *Bacteroides* and *Ruminococcus gnavus,* both of which are known to be associated inflammatory bowel disease[20,21]. Other members of LGC bacteria include *Parabacteroides, Campylobacter, Dialister, Porphyromonas, Staphylococcus* and *Anaerostipes.* In addition, few of the key bacterial metabolites in LGC individuals include modules for β-glucuronide degradation, degradation of aromatic amino acids, and dissimilatory nitrite reduction, all of which are known to have deleterious effects.

Overall, the healthy gut microbiota is predominantly constituted by the phyla Firmicutes and Bacteroidetes. This is followed by the phyla Actinobacteria and Verrucomicrobia. Even though this general profile remains constant, gut microbiota exhibits both temporal and spatial differences in distribution at the genus level and beyond. As one travels from the esophagus distally to the rectum, there will be a marked difference in diversity and number of bacteria ranging from 10^1 per gram of contents in the esophagus and stomach to 10^{12} per gram of contents in the colon and distal gut[22]. Figure 2 depicts the temporal diversity of the gut microbiota as one travels from the esophagus distally to the colon. *Streptococcus* appears to be the dominant genus in the distal esophagus, duodenum and jejunum[23,24]. *Helicobacter* is the dominant genera

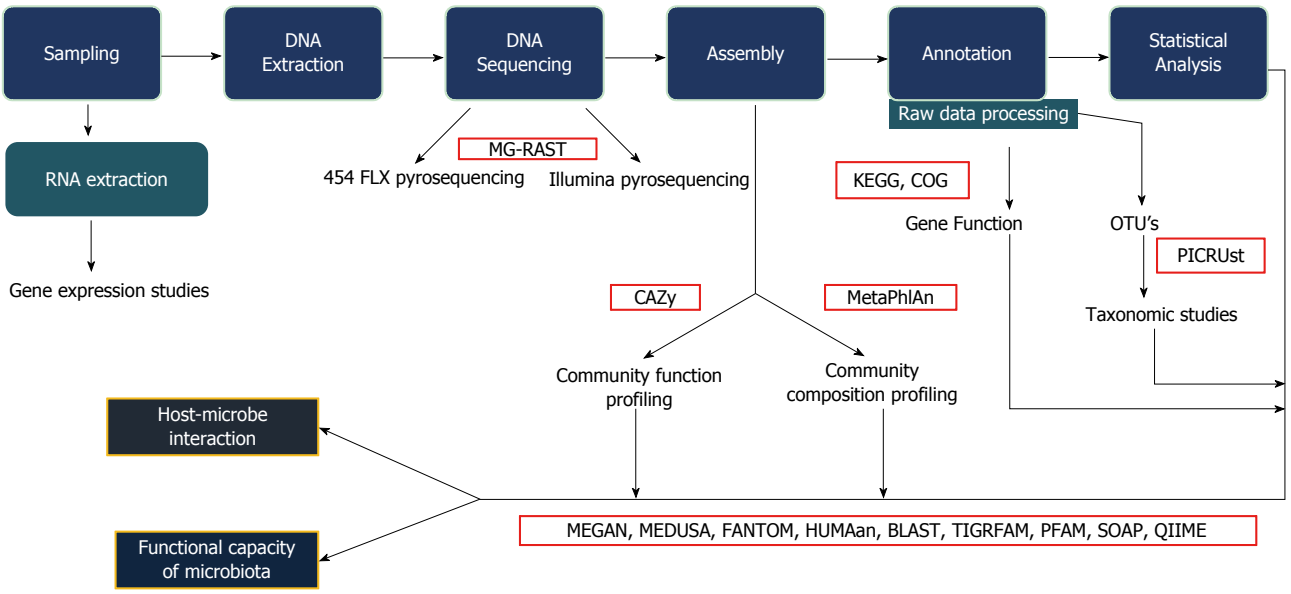

Figure 1 Bioinformatics work flow. This figure explains the various steps involved in the bioinformatics analysis, starting from collection of samples, extraction, sequencing and statistical analysis. The interaction between host and microbes along with the functional capacity of the microbiota can be studied. MG-RAST: Metagenomics rapid annotation using subsystem technology; CAZy: Carbohydrate active-enzymes; MetaPhlAn: Metagenomic phylogenetic analysis; KEGG: Kyoto encyclopaedia for genes and genomics; COG: Clusters of orthologous group; PICRUst: Phylogenetic investigation of communities by reconstruction of unobserved states; MEGAN: Meta genome analyzer; MEDUSA: Metagenomic data utilization and analysis; FANTOM: Functional annotation and taxonomic analysis of metagenomes; HUMAan: Human microbiome project unified metabolic analysis network; BLAST: Basic local alignment search tool; TIGRFAM: Protein sequence classification; PFAM: Protein families; SOAP: Short oligonucleotide analysis package; QIIME: Quantitative insights into microbial ecology.

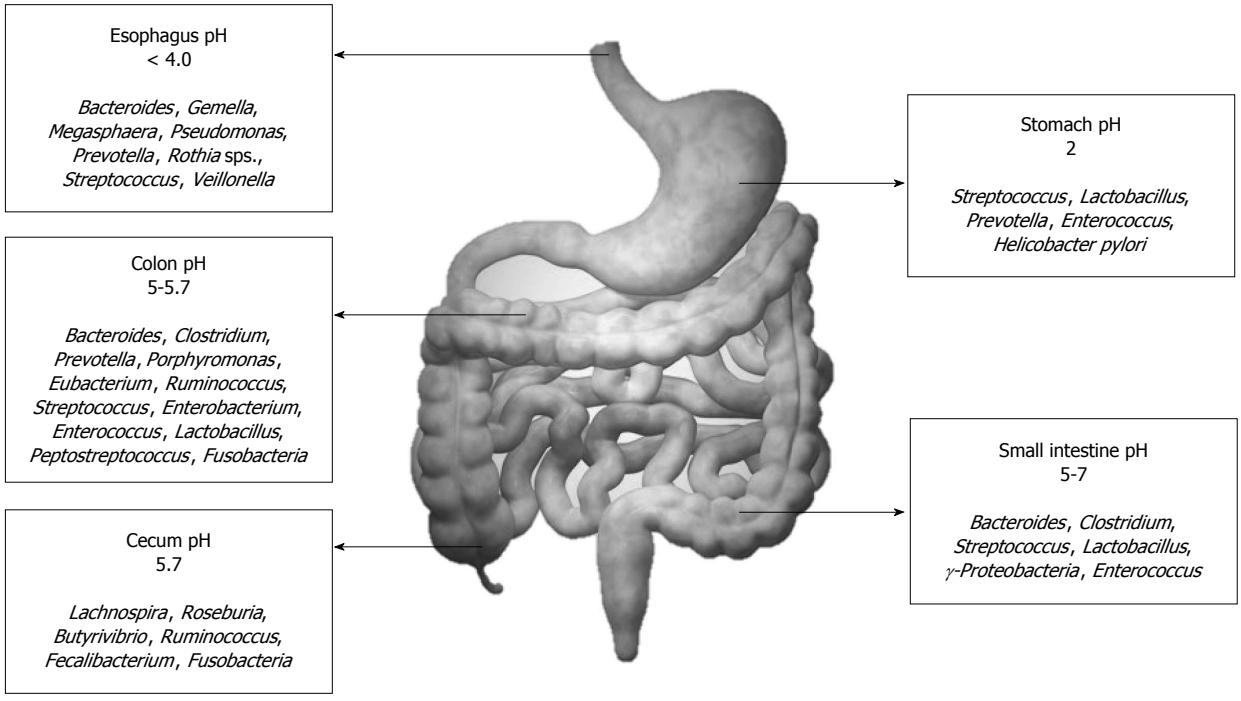

Figure 2 Distribution of the normal human gut flora.

present in the stomach and determines the entire microbial landscape of the gastric flora, *i.e.*, when *Helicobacter pylori* (*H. pylori*) inhabits the stomach as a commensal, there is a rich diversity constituted by other dominant genus such as *Streptococcus* (most dominant), *Prevotella*, *Veillonella* and *Rothia*[25,26]. This diversity shrinks once *H. pylori* acquire a pathogenic phenotype. The large intestine constitutes of over 70% of the all microbes found in the body, and gut flora that is generally discussed in the context of disease state by and large implies the colonic flora (especially those derived from stool metagenomic data). The

predominant phyla that inhabit the large intestine include Firmicutes and Bacteroidetes. Traditionally, the Firmicutes: Bacteroidetes ratio has been implicated in predisposition to disease states[27]. However, the significant variability even in healthy individuals that has been observed across recent studies makes the relevance of this ratio debatable. Besides genera from phyla Firmicutes and Bacteroidetes, human colon also harbors primary pathogens, *e.g.*, species such as *Campylobacter jejuni*, *Salmonella enterica, Vibrio cholera and Escherichia coli* (*E. coli*), *and Bacteroides fragilis,* but with a low abundance (0.1% or less of entire gut microbiome)[6,28]. The abundance of the phylum *Proteobacteria* is markedly low; and its absence along with high abundance of signature genera such as *Bacteroides, Prevotella* and *Ruminococcus* suggests a healthy gut microbiota[29]. Besides this longitudinal difference, there also exists an axial difference from the lumen to the mucosal surface of the intestine. While *Bacteroides, Bifidobacterium, Streptococcus, Enterobacteriacae, Enterococcus, Clostridium, Lactobacillus* and *Ruminococcus* are the predominant luminal microbial genera (can be identified in stool), only *Clostridium, Lactobacillus, Enterococcus and Akkermansia* are the predominant mucosa and mucus associated genera (detected in the mucus layer and epithelial crypts of the small intestine)[30].

The other way of classifying the gut flora, as proposed by the MetaHIT Consortium[31], is based on species composition which cluster into well-balanced host-microbial symbiotic states that is stable over geography and gender, but can respond differently to diet and drugs. These clusters have been named enterotypes. Interestingly, the abundance of molecular functions however may not correlate with abundance of species within the enterotypes. Furthermore, as shown in a recent study on the association of gut microbiome with atherosclerosis, there may not be significant changes in the enterotype observed in disease conditions[32]. There are broadly three enterotypes[29], namely: Enterotype 1, which has a high abundance of *Bacteroides*; Enterotype 2, which has high abundance of *Prevotella*; and Enterotype 3 which has high abundance of *Ruminococcus*. The bacteria belonging to Enterotype 1 have a wide saccharolytic potential, as evidenced by the presence of genes that code for enzymes such as proteases, hexoaminidases and galactosidases. In view of these set of enzymatic potential, it appears likely that these organisms derive energy from dietary carbohydrates and proteins. Enterotype 2 behave predominantly as a degrader of the mucin glycoproteins that line the gut mucosal layer. Enterotype 3 also is associated with mucin degradation, in addition to membrane transport of sugars. The enterotypes also possess other specific metabolic functions. For instance, biotin, riboflavin, pantothenate and ascorbate synthesis are more abundantly seen in enterotype 1 while thiamine and folate synthesis are more predominant in enterotype 2. However, the concept of enterotyping does not explain the relative distribution of different classes of organisms in different individuals. Since *Bacteroides* and *Prevotella* do not exist in equal proportion in the gut, the concept of enterogradient based upon the dominance of either of these two organisms could be another defining concept. This could explain the inter-individual distribution at the class level in a better way[33].

FUNCTIONAL ASPECTS OF THE NORMAL GUT MICROBIOTA

The gut microbiota maintains a symbiotic relationship with the gut mucosa and imparts substantial metabolic, immunological and gut protective functions in the healthy individual. The gut microbiota, which derives its nutrient from host dietary components and shed epithelial cells, is an organ by itself with an extensive metabolic capability and substantial functional plasticity[34]. These characteristics of the gut microbiome have been rapidly shifting the research focus from the abundance and diversity of the microbial members to the functional aspects. This section provides a brief overview of the major functions of the normal gut microbiota.

Nutrient metabolism

The gut microbiota largely derives their nutrients from dietary carbohydrates. Fermentation of the carbohydrates that escaped proximal digestion and indigestible oligosaccharides by colonic organisms such as *Bacteroides, Roseburia, Bifidobacterium, Fecalibacterium,* and *Enterobacteria* result in the synthesis of short chain fatty acids (SCFA) such as butyrate, propionate and acetate, which are rich sources of energy for the host[35,36]. This host energy balance is believed to be mediated *via* a ligand-receptor interaction of the SCFAs with a G protein-coupled receptor Gpr41. Another enteroendocrine hormone PYY (Peptide Tyrosine Tyrosine/Pancreatic Peptide YY3-36) has also been implicated in this action[37]. Furthermore, butyrate can prevent the accumulation of toxic metabolic by-products such as D-lactate[38]. Members of the genus *Bacteroides,* which are the predominant organisms that participate in carbohydrate metabolism, perform this by expressing enzymes such as glycosyl transferases, glycoside hydrolases and polysaccharide lyases. The best example among these organisms is *Bacteroides thetaiotaomicron* that is endowed with a genome that codes for over 260 hydrolases, which is far more than the number encoded by the human genome[39]. The oxalate that is synthesized in the intestine as a result of carbohydrate fermentation and bacterial metabolism is countered by organisms such as *Oxalobacter formigenes, Lactobacillus species,* and *Bifidobacterium species* thereby reducing the risk of formation of

oxalate stone in the kidney[40,41].

The gut microbiota has also been shown to impart a positive impact on lipid metabolism by suppressing the inhibition of lipoprotein lipase activity in adipocytes. Furthermore, *Bacteroides thetaiotaomicron* is demonstrated to augment the efficiency of lipid hydrolysis by up regulating expression of a colipase that is required by pancreatic lipase for lipid digestion[42].

The gut microbiota is also enriched with an efficient protein metabolizing machinery that function *via* the microbial proteinases and peptidases in tandem with human proteinases. Several amino acid transporters on the bacterial cell wall facilitate amino acid entry from the intestinal lumen into the bacteria, wherein several gene products convert the amino acids into small signaling molecules and antimicrobial peptides (bacteriocins). Important examples include conversion of L-histidine to histamine by the bacterial enzyme histamine decarboxylase, which is coded by the bacterial *hdcA* genes[43]; and glutamate to γ-amino butyric acid (GABA) by glutamate decarboxylases, which are coded by the bacterial *gadB* genes[44].

Synthesis of vitamin K and several components of vitamin B is another major metabolic function of the gut microbiota. Members of genus *Bacteroides* have been shown to synthesize conjugated linoleic acid (CLA) that is known to be antidiabetic, antiatherogenic, antiobesogenic, hypolipidemic and have immunomodulatory properties[45-47]. The gut microbiota, especially *Bacteroides intestinalis*, and to a certain extent *Bacteroides fragilis* and *E. coli*, also has the capacity to deconjugate and dehydrate the primary bile acids and convert them into the secondary bile acids deoxycholic and lithocolic acids in the human colon[48]. The normal gut microbiota has also been shown to impart a healthy metabolome in the serum by increasing the concentrations of pyruvic acid, citric acid, fumaric acid and malic acid, all of which are indicators of higher energy metabolism[49].

Recent studies have shown that human gut microbiota is also involved in breakdown of various polyphenols (phenolic compounds) that are consumed in the diet. Polyphenolic secondary metabolites are found in a variety of plants, fruits and plant derived products (tea, cocoa, wine), for example, flavanols, flavanones, flavan-3-ols, anthocyanidins, isoflavones, flavones, tannins, lignans and chlorogenic acids. Of these, flavanoids and flavanoid sub-families are most commonly absorbed by the intestine. Polyphenols exist as glycosylated derivatives bounded with sugars such as glucose, galactose, rhamnose, ribulose, arabinopyrinose and arabinofuranose. Polyphenols, which usually remain inactive in diet are biotransformed to active compounds after removal of the sugar moiety by the gut microbiota, among other factors. Structural specificity of polyphenol and individual richness of microbiota determines the level of biotransformation that occur in the intestine. The final active products are absorbed by the portal vein and travel to other tissues and organs, thereby providing antimicrobial and other metabolic action. This can be exemplified by the conversion of inactive isoflavones to the aglycon equol, which has anti-androgenic and hypolipidemic effects[50]. Table 2 shows an elaborate list of the dietary polyphenols and the gut microbiota involved in its transformation[51-69].

Xenobiotic and drug metabolism

The capability of the gut microbiome to metabolize xenobiotics and drugs was first recognized over 40 years back. An increasing body of evidence has now provided sufficient insights on the role of the gut microbiota on xenobiotic metabolism, which could have profound impact on therapy for various diseases in future. Recent studies by Clayton *et al*[70] have shown that a gut microbial metabolite p-cresol can reduce the capacity of the liver to metabolize acetaminophen due to competitive inhibition of hepatic sulfotransferases. Furthermore, cardiac glycosides like digoxin have been recently shown to up-regulate a cytochrome containing operon in the common organism *Eggerthella lenta* from the Actinobacteria phyla, which results in inactivation of digoxin[71]. Another interesting example of microbiome induced drug metabolism is the microbial β-glucoronidase induced deconjugation of the anticancer drug irinotecan that can contribute to its toxicities such as diarrhea, inflammation and anorexia[72].

Antimicrobial protection

The requirement of a healthy gut microbiota for normal homeostasis puts the gut mucosal immune system in a challenging situation in that it needs to be tolerant to the beneficial commensals and yet prevent overgrowth of the resident pathogens. One of the simplest mechanisms of antimicrobial protection is the presence of the two-tiered mucus layer, which keeps luminal microbes away from epithelial contact, predominantly in the large intestine. Mucus is constituted of a variety of mucin glycoproteins that are secreted by the intestinal goblet cells and extend up to 150 μm away from the colonic epithelium[73,74]. The inner layer is denser and does not contain any organism, while the outer layer is more dynamic and provides glycans as a source of nutrition for the organisms[75]. Other than the mucin glycoproteins, the goblet cells also produce factors like trefoil-factor and the resistin-like molecule-β that can stabilize mucin polymers and thereby maintain barrier integrity[76,77].

Contrary to the large intestine where the mucus plays an important role, antimicrobial proteins play a larger role in the small intestine since the mucus layer here is discontinuous and inadequate. The gut microbiota, *via* its structural components and metabolites, has been shown to induce synthesis of antimicrobial proteins (AMP) such as cathelicidins, C-type lectins, and (pro)defensins by the host Pa-

Table 2 Types of dietary polyphenols present in various foods and the types of microorganisms those are responsible for the degradation

Polyphenolic compounds	Classes involved	Foods containing polyphenols	Gut bacteria
Flavanols	Kaempferol[51], Quercetin[53], Myricetin[52]	Onions, capers, apples, broccoli, grapes and plums	*Bacteroides distasonis*, *Bacteroides uniformis*, *Enterococcus casseliflavus* and *Eubacterium ramulus*
Flavanones	Hesperetin, Naringenin[54]	Citrus fruits and tomatoes	*Clostridium* sps, *E. ramulus*
Flavan-3-ols	Catechin[55], Epicatechin[56], Gallocatechin[57,58]	Green tea, cocoa, kola, banana, pomegranate	*Bifidobacterium infantis* and *Clostridium coccides*
Anthocyanidins	Cyanidin[59], Pelagonidin, Malvidin[60]	Bilberries and all red, blue and purple fruits (especially berries)	*Lactobacillus plantarum*, *L. casei*, *L. acidophilus* and *Bifidobacterium longum*
Isoflavones	Daidzein[61,62], Geinstein[63], Formononentin[64]	Soy, beans, lentils, chickpea (Fabaceae family)	*Lactobacillus* and *Bifidobacterium*
Flavones	Luteolin[65], Apigenin[66]	Cereals, parsley, thyme, celery and citrus fruits	*C.orbiscinden*, *Enterococcus avium*
Tannins	Gallo tannins, Ellagitannins[67]	Raseberries, cranberries, strawberries, walnuts, grapes and pomegranate	*Butyrivibrio* sps
Lignins	Secoisolariciesinol, metaresinol, pinoresinol, larciresinol, isolarciresinol, syringiresinol[68]	Flax seeds, cereals, strawberries, and apricots	Species of *Bacteroides*, *Clostridium*, *Peptostreptococcus* and *Eubacterium*
Chlorogenic acids	Caffeic acid, feruic acid[69]	Peach, plums and coffee	*E. coli*, *Bifidobacterium* sps and *L. gasseri*

neth cells *via* a pattern recognition receptor (PRR) mediated mechanism[78,79]. The PRR family includes the membrane associated TLRs, C-type lectin receptors (CLRs) such as Dectin-1, and the cytosolic nucleotide-binding and oligomerisation domains (NOD) like receptors (NLRs)[80]. The PRRs in turn are activated by organism specific microbe-associate molecular patterns (MAMPs), which includes various microbial components such as peptidoglycan, LPS, lipid A, flagella and bacterial RNA/DNA, fungal cell wall β-glucans[80,81]. PRR-MAMP (pattern recognition receptor- Microbe Associated Molecular Patterns) cross-talk results in activation of several signaling pathways that are essential for promoting mucosal barrier function, and production of AMPs, mucin glycoproteins and IgA. Since the Paneth cells reside in the base of the small intestinal crypts, concentration of the AMPs are maximal at this location. Even though the composite healthy microbiota appears to be a prerequisite for AMP production, *Bacteroides thetaiotaomicron* and *Lactobacillus innocua* appear to be among the key individual species that drive this production[82,83]. The organism *Bacteroides thetaiotaomicron* has also been shown to induce expression of the matrix metalloproteinase matrilysin from the Paneth cells, which subsequently cleaves prodefensin to form active defensin[84]. Another example of microbiota-host interaction in providing antimicrobial protection is the capability of *Lactobacillus* sp. to produce lactic acid, which can augment the antimicrobial activity of host lysozyme by disrupting the outer membrane of the bacterial cell wall[85]. Besides this two-way interactive mechanism of AMP expression, bacterial metabolic products such as SCFAs and lithocholic acid have also been shown to induce the expression of cathelicidin by mechanisms involving histone deacetylation and MEK/ERK (Mitogen activated protein kinase/Extracellular signal regulated kinases) pathway[86-88]. The AMPs primarily act by disrupting the surface structures of both commensals and pathogens.

The other mechanism that the gut microbiota has evolved is to keep a check on the overgrowth of pathogenic strains by inducing local immunoglobulins. The gut microbiota, especially Gram-negative organisms like *Bacteroides* are shown to activate intestinal dendritic cells (DCs), which induces plasma cells in the intestinal mucosa to express secretory IgA (sIgA)[89]. The sIgA can in turn coat the gut microbiota. The sIgA that coats the microbiota are predominantly of sIgA2 subclass, which is more resistant to degradation by bacterial proteases. Furthermore, the intestinal epithelial cells (IECs) can produce a proliferation-inducing ligand (APRIL) in a TLR-mediated bacterial sensing mechanism that can induce class switching from a systemic sIgA1 phenotype to the intestinal mucosal sIgA2[90]. These mechanisms restrict the translocation of the microbiota from the intestinal lumen to the circulation, thereby preventing a systemic immune response.

Immunomodulation

The gut microbiota contribute to gut immunomodulation in tandem with both the innate and adaptive immune systems. The components and the cell types from the immune system that participate in the immunomodulatory process includes the gut associated lymphoid tissues (GALT), effector and regulatory T cells, IgA producing B (plasma) cells, Group 3 innate lymphoid cells, and, resident macrophages and dendritic cells in the lamina propria (Figure 3).

The role of gut microbiota in shaping a normal GALT is implied by the impaired development of the Peyer's patches and isolated lymphoid follicles that are marked by the abundance of IgE^+ B cells instead of the normally seen IgA^+ B cells[91]. The effector T cell responses in the intestine have also been shown to be

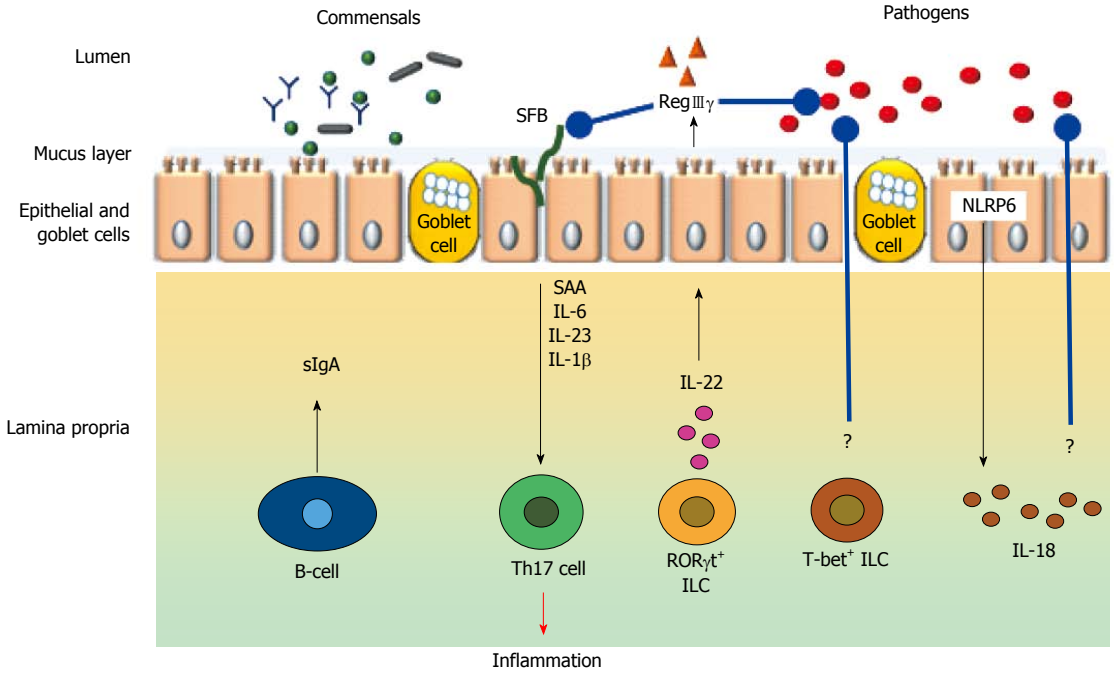

Figure 3 Broad schematic representation of cell types and mediators involved in immunomodulation in the gut. Black arrow indicate either physiological secretion or activation; Red arrow indicates pathological event; Blue arrows with rounded ends indicates pathogen inhibition; ? indicates unknown mechanisms; SFB indicates short filamentous bacteria.

primarily controlled by Th2 responses as opposed to the Th1 responses[92]. The latter is primarily mediated by Th1 and Th17 cells under a physiological milieu; and gut commensals are believed to result in TLR-MyD88 signaling mediated activation of IL1β which in turn promote development of IL17[93].

Intestinal microbiota is also essential for the normal development and function of Foxp3$^+$ T regulatory (Treg) cells. However, the mechanism by which this is mediated is still not clear. For example, in the case of certain *Clostridium* clusters it could be either independent of PRRs or dependent on My-D88 dependent mechanisms[94]. In the case of *Bacillus fragilis,* induction of Tregs appear to be mediated by TLR2 signaling by polysaccharide A[95]. SCFAs, especially butyrate have also been implicated in the development and function of Tregs. SCFAs are shown to activate G-protein coupled receptors expressed by the IECs and regulate Treg by epigenetic regulation (increased acetylation) of the *Foxp3* locus[96-98].

As mentioned in the previous section, mucosal plasma cells produce secretory IgA upon induction by DCs. Though the mechanisms are not clear, it is speculated that this function is mediated by My-D88 signaling in lamina propria and follicular DCs. My-D88 signaling can be activated by the gut microbiota. Furthermore, in addition to class switching of sIgA by APRIL mediated stimulation, the gut microbiota also stimulate DCs in the Peyer's patches to secrete TGF-β, CXCL13, and B-cell activating protein (BAFF), which leads to IgA production and class switching[99].

Another set of innate immune cells, namely the innate lymphoid cells (ILCs) are capable of responding rapidly to epithelium-derived cytokine signals[100]. ILCs arise from common lymphoid precursors and have a cytokine expression pattern that is similar to that of T helper subsets (particularly Th17 cells); but the differentiation is more dependent on microbial composition rather than somatic recombination[101]. Based on the functional properties, ILCs can be divided into three groups, namely, group 1 [T box expressed in T cells (T-bet)$^+$], Group 2 [Gata binding protein 3 (GATA-3)$^+$], and group 3 [retinoid-related orphan receptor gamma t (RORγt)$^+$]. Of these, RORγt$^+$ ILCs appear to be most closely associated with regulation of gut immunity[102]. Even though the precise mechanisms are unclear, it is speculated that gut microbes could regulate ILCs both directly and indirectly. Evidence in favor of the former is provided by the observation that the bacterial metabolite indole-3-aldehyde stimulates ILC *via* the aryl hydrocarbon receptor to induce synthesis of IL22[103]. Indirect mechanism of ILC regulation, on the other hand, is *via* the recruitment of other immune cells such as the CX$_3$CR1$^+$ intestinal macrophages[104].

The immunomodulatory action of resident macrophages in the lamina propria is to express pro-IL1β in the steady state, which aids in the rapid production of mature IL1β in response to pathogen invasion. MyD-88 dependent mechanisms induced by commensal flora is essential for this action; while the microbiota regulated IL-10 production by the macrophages entail MyD-88 independent mechanisms[105,106].

Apart from the gut microbiota, other factors also

play a role in modulation of the gut immune system. For example, the IECs secrete an isoform of alkaline phosphatase (intestinal alkaline phosphatase) that dephosphorylates the LPS endotoxin[107]. Another example is the reduced neutrophil recruitment into the intestinal lumen in response to tumour necrosis factor-α (TNF-α). This action is mediated by the intestinal alkaline phosphatase[107]. Furthermore, an immunoprotective mechanism that is acquired at birth and is seen predominantly with vaginal delivery is the down regulation of IL-1 receptor-associated kinase (IRAK-1), which acts through TLR4[108].

Integrity of the gut barrier and structure of the gastrointestinal tract

Currently there is a convincing body of evidence that supports the role of the gut microbiota in maintaining the structure and function of the gastrointestinal tract. *Bacteroides thetaiotaomicron* is reported to induce expression of the small proline-rich protein 2A (sprr2A), which is required for maintenance of desmosomes at the epithelial villus[109]. Another mechanism that maintains the tight junctions is by TLR2 mediated signaling that is stimulated by the microbial cell wall peptidoglycan[110]. Furthermore, the *Lactobacillus rhamnosus GG* strain produces two soluble proteins namely p40 and p75 that can prevent cytokine induced apoptosis of the intestinal epithelial cells in an epithelial growth factor receptor (EGFR) and protein kinase C (PKC) pathway dependent manner[111]. The endocannabinoid system is yet another entity that regulates gut microbiota mediated maintenance of the gut barrier function. *E.g.*, the Gram negative bacteria *Akkermansia muciniphilia* can increase the levels of endocannabinoids that control gut barrier functions by decreasing metabolic endotoxemia[112].

The gut microbiota contributes to structural development of the gut mucosa by inducing the transcription factor angiogenin-3, which has been implicated in the development of intestinal micro-vasculature[113]. This is also supported by a significant reduction of villus capillary network in germ-free (GF) mice, which in turn can impair nutrient digestion and absorption. Other evidence that support role of gut microbiota in maintaining structure and function is obtained from GF mice that have a lower intestinal surface area[114], thin villi (secondary to lower re-generation)[115], increase cell cycle time[116] and im-paired peristalsis[117]. The gut microbiota can also modulate mucosal glycosylation patterns that are microbial attachment sites both at the cell surface and subcellular levels. For example, a signaling molecule secreted by the organism *Bacteroides thetaiotaomicron* can stimulate expression of the carbohydrate moiety fucose on the cell surface glycoconjugates[118].

FACTORS AFFECTING VARIATIONS IN THE NORMAL GUT MICROBIOTA

Several factors contribute to the shaping of the healthy gut microbiota; and this continues dynamically all throughout the life of an individual.

Age

Even though it is widely believed that the gut gets colonized by microbes immediately after birth, there is emerging evidence that the infant gut could be colonized by organisms even *in utero*[119]. 16S rRNA based sequencing studies have revealed that the first meconium is rich in genera such as *Escherichia-Shigella*, *Enterococcus*, *Leuconostoc*, *Lactococcus*, and *Streptococcus*[120]. Nevertheless, it is now clear that the first microbiota profile is largely shaped by the mode of delivery. The intestines of infants born vaginally are initially colonized by organisms from the maternal vagina, which is best exemplified by the organisms from the genera *Lactobacillus* and *Prevotella*[121]. On the contrary, in cesarean delivery mostly the maternal skin flora colonizes the infant's intestine, as exemplified by the dominance of *Streptococcus, Corynebacterium,* and *Propionibacterium*[119,121]. The initial milieu of the infant's gut microbiota after primary inoculation appears unstable and devoid of diversity; but with time it stabilizes, diversifies, and acquires 40%-60% similarity with the adult microbiota by the age of 3 years[122]. On the contrary, studies have also shown that young children and adolescents could demonstrate significant differences in proportions of *Bacteroides* and *Bifidobacterium* compared to adults[123,124]. The gut microbiota by and large rest in a stable state from the 3rd to the 7th decade of life, even though proportions of *Bifidobacteria*, Firmicutes, and *Fecalibacterium prausnitzii* tend to decrease with an increase in *E. coli,* Proteobacteria and *Staphylococcus*[125-127]. Few of the functional impacts of the temporal alteration in the normal gut flora include a reduced capability to synthesize vitamin B_{12}, reduced activities of microbial reductases, increased tendency for DNA alterations, elevated stress response, and immune dysfunction[128]. Although the initially developing microbiota is largely influenced by the type of feed (breast milk or formula feeds) after primary inoculation, the temporal alte-ration is affected by dietary patterns, lifestyle, life events, and environmental factors including antibiotic use[1].

In pre-term infants, bacteria that colonize the gut include *Bifidobacterium* and *Lactobacillus* and basically, these differ depending on the type of feeding habits. In formula-fed infants, *Enterococcus*, *Enterobacteria*, *Bacteroides*, *Clostridia*, and other anaerobic *Strep-tococcus* dominates the gut niche; whereas, in

breast-fed infants *Bifidobacterium* and *Lactobacillus dominates.* Breast milk contains indigestible glycans termed as human milk oligosaccharides (HMO) which are easily broken down by these bacteria. Pre-term microbiota are said to maintain the gut associated lymphoid tissue (GALT), and is involved in generating the innate immunity during development. Therefore, abnormal colonization of the gut microbiota may result in pediatric diseases because of poor immunity[129,130].

Diet

The earliest effect on the gut microbiota, after the mode of delivery, is the early infant diet, *i.e.*, breast milk and formula feeds. Several studies have shown substantial differences in the gut microbial composition between breast-fed and formula-fed infants. It is important to understand the effect of breast milk and formula feeds on the gut microbiota since there has been an increasing trend of moving away from breast-feeding by modern day mothers. Besides meeting the nutritional and physiological demands of the infant, breast milk also contains several bioactive compounds that are not available in formula-feeds. These compounds have a significant role in nutrient digestion and absorption, immune protection and anti-microbial defense[131,132]. HMOs provide nutrition to the colonic bacteria of the infant, thereby providing a selective growth advantage for *Bifidobacterium* sp.[133]. This has been observed at a significantly higher abundance in breast-fed infants compared to that in formula-fed infants. These organisms ferment dietary oligosaccharides resulting in health promoting SCFAs such as butyrate, and modulate the host immune system to express IgG[134]. Studies have shown that several strains of *Bifidobacterium,* especially the *Bifidobacterium longus* subs *infantis* contain unique gene cluster (HMO-related gene cluster 1) that codes for difference glycosidases (sialidase, fucosidase, hexosaminidase and galactosidase) and carbohydrate transporters that are capable of importing and metabolizing HMOs[134]. On the contrary, the abundance of anaerobic organisms like *Bacteroides* sp. and *Clostridium* sp. is lower in breast-fed infants as compared to formula-fed ones[135-137]. Even though *Bacteroides* sp. can also digest HMO, the abundance of *Bifidobacterium* is higher in breast-fed infants, thus pointing towards competitive relationship between these two organisms in favor of *Bifidobacterium* in breast-fed infants.

Diet continues to be the most important determinant in shaping the composition, diversity and richness even throughout adulthood. In general, intake of diet rich in fruits, vegetables and fibers is associated with a higher richness and diversity of the gut microbiota. Individuals consuming this kind of a diet have a higher abundance of the insoluble carbohydrate metabolizing organisms of the Firmicutes phylum such as *Ruminococcus bromii, Roseburia* and *Eubacterium rectale*[138]. It was recently shown that a 4-d administration of animal-based diet resulted in a decrease in the abundance of Firmicutes; and an increase in that of bile-tolerant organisms such as *Alistipes* sp. and *Bacteroides* sp. from the phylum Bacteroidetes and *Bilophila* sp. from the phylum Proteobacteria. This indicates that even very short dietary manipulations can have substantial impact on the gut microbiota[139].

Several studies have shown that there are significant geographic and seasonal variations in the gut microbiome. However, these differences were also associated with a difference in dietary patterns. For example, it was demonstrated that rural African children had a higher abundance of *Prevotella*, while children from Europe had higher proportions of *Bacteroides*[140]. Even though *Prevotella* and *Bacteroides* are taxonomically and functionally similar, higher abundance of *Prevotella* indicates an agrarian diet that was consumed by the African children. On the contrary, the children from Europe consumed a western diet rich in animal protein, sugar, starch and poor in fibers, which is marked by the higher abundance of *Bacteroides.* Furthermore, it was also shown that the relative abundance of the phylum Actinobacteria was significantly higher in Hutterites during winter season compared to that during summers. This could be ascribed to the higher intake of meat-based diet in winter when compared to the fresh, carbohydrate and fiber rich diet that was consumed during summer[141].

Dietary polyphenols, besides their systemic antimicrobial and metabolic functions, also play a role in the inhibition of gut bacteria. While the polyphenolic compound querectin is degraded by *Bacteroides distasonis*, *Bacteroides uniformis*, *Bacteroides ovatus*, *Enterococcus casseliflavus*, and *Eubacterium ramulus*are the compound that degrade this flavanol, hesperetine (a rutinoside containing aglycon), is poorly degraded by the colonic microbiota. This aglycon has an inhibitory activity against vancomycin- intermediate *Staphylococcus aureus* and *H. pylori*[142].

Seaweeds are active resources with bioactive compounds with various biological activities such as antibacterial, anti-oxidant, anti-inflammatory, anti-coagulant, anti-viral and apoptotic activity. They are rich source of fiber with nearly 50%-60% of water soluble fibers, and are also rich in sulfated polysaccharides such as porphyrans, and agarases. Few species of red sea weeds like *Palmaria decipiens* and *Pterocladiella capillacea* contains sulfated polysaccharides and uronic acids (*i.e.*, xylans and xylogalactans) respectively[143]. Several human and rat studies have demonstrated a significant shift in the gut microbiota upon the use of seaweeds as a food supplement. In humans, supplementation of *Gelidium* seaweed has significantly increased the expression of *Bifidobacterium* genera, without any change in the

others. There was also an increase in the production of the SCFA's[144]. Another study conducted on Japanese populations explained the transfer of porphyranases and agarases to the gut bacteria *Bacteroides plebius* through carbohydrate active enzymes (CAZymes)[145]. These studies points towards the feasibility the use of sea weeds as a potential prebiotic.

Antibiotics

Even though study on antibiotics in general have centered around their bactericidal and bacteriostatic activities against pathogens, recent years have seen several studies on their effect on gut bacterial ecology in a holistic manner. A strong body of evidence has now clearly demonstrated that use of antibiotics does have several short and long-term implications in the ecology of the normal gut microbiota. It has been shown that multi-drug resistant bacterial genes have been prevalent for thousands of years before the advent of antibiotics, indicating an influence of exposure to small molecules from the environment with growth inhibitory properties[146]. This could also be secondary to a dysbiotic commensal microbiota that could further augment the development of resistance genes[147]. This culminates in uncoupling of the mutualistic relationship between the healthy gut microbiota and the host intestinal milieu.

One of the major properties of the healthy gut microbiota against pathogen is the capability to cause competitive exclusion[148]. It was demonstrated around four decades ago that antibiotics could result in disruption of the competitive exclusion machinery that resulted in *Salmonella* infection immediately after antibiotic therapy. One of the possible mechanisms of this kind of event could be a loss of the wide network of interspecies interactions within the microbiota that increase the abundance of host-derived sialic acid which is growth promoting for pathogens such as *Salmonella typhimurium* and *Clostridium difficile*[149]. Major changes in the gut microbiota in response to antibiotics include diminished taxonomic diversity and persistence of the changes in a substantial proportion of individuals. It has been shown that the effect of even short-term use (7 d) of broad-spectrum antibiotics with predominant anaerobic coverage (*e.g.*, Clindamycin) could last up to 2 year, with a persistent non-recovery of the diversity of *Bacteroides*[150]. Similarly, a short course *H. pylori* eradication with clarithromycin containing triple therapy resulted in a dramatic reduction in the diversity of *Actinobacteria* with a thousand-fold increase in the *ermB* resistance gene[151]. This persisted for over 4-years in a proportion of these patients, while it recovered in the others. The effect of ciprofloxacin, which has predominantly Gram-positive coverage, is relatively short-lived with abrupt reduction of *Ruminococcus* sps.[152]. Another recent study that evaluated the role of short course (7 d) of ciprofloxacin and beta-lactams indicated the reduction of microbial diversity by 25% and the core taxa from 29 to 12 with an increase in the Bacteroidetes: Firmicutes ratio[153]. The major concern that stems out of use of broad-spectrum antibiotics, besides alteration of the normal gut microbial diversity, is the phenomenon of propagating the resistance strain *via* horizontal gene transfer[154,155]. Bacterial species are capable of transferring mutant genetic information across different species through mechanisms such as conjugation, phage transduction and natural transformation. The gene transfer could also be *via* transposons and integrin. Interestingly, it has been shown that among different environments, the human gut associated microbiota has 25 times more likelihood of having horizontal gene transfer[156]. This would result in development of a reservoir state of resistance genes, and therefore mandates extreme care in the use of broad spectrum antibiotics.

PROBIOTICS, PREBIOTICS AND SYNBIOTICS

The World Health Organization defines probiotics as live microorganisms that can provide benefits to human health when administered in adequate amounts. Several species such as *Lactobacillus casei, Lactobacillus planatarum, Lactobacillus bulgaricus, Lactobacillus acidophilus, Bifidobacterium longum, Bifidobacterium infantis, Streptococcus thermophilus, E. coli* strain Nissle 1917, to name a few have been shown to impart immunomodulatory and gut barrier functions. These and several others have been used commercially in the management of human illnesses *e.g.*, IBD and antibiotic associated diarrhea. The fundamental concept of using these organisms in the treatment armamentarium is mimicking the physiological health promoting functions of the "good" bacteria. Addition of a prebiotic could possibly augment the effect of the probiotics. Prebiotics are defined as food ingredients that contain non-digestible oligosaccharides (*e.g.*, galactooligosaccharides and inulin); and a probiotic and prebiotic are together called a synbiotic. The gut bacteria selectively ferment these fibers resulting in the synthesis of SCFAs, which in turn imparts the pro-health effects (*vide supra*). A detailed discussion on pro- and prebiotics is out of the scope of this review since it deals predominantly on a normal gut microbiota. Nevertheless, we believe that even though dietary fibers and healthy gut microbiota are known to promote health, use of synbiotics for maintenance of health needs to be studied with much robustness before using them commercially as health promoters[157,158].

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MINIREVIEWS

Microscopic colitis: A review of etiology, treatment and refractory disease

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Abstract

Microscopic colitis is a common cause of chronic, nonbloody diarrhea. Microscopic colitis is more common in women than men and usually affects patients in their sixth and seventh decade. This article reviews the etiology and medical management of microscopic colitis. The etiology of microscopic colitis is unknown, but it is associated with autoimmune disorders, such as celiac disease, polyarthritis, and thyroid disorders. Smoking has been identified as a risk factor of microscopic colitis. Exposure to medications, such as non-steroidal anti-inflammatory drugs, proton pump inhibitors, and selective serotonin reuptake inhibitors, is suspected to play a role in microscopic colitis, although their direct causal relationship has not been proven. Multiple medications, including corticosteroids, antidiarrheals, cholestyramine, bismuth, 5-aminosalicylates, and immunomodulators, have been used to treat microscopic colitis with variable response rates. Budesonide is effective in inducing and maintaining clinical remission but relapse rate is as high as 82% when budesonide is discontinued. There is limited data on management of steroid-dependent microscopic colitis or refractory microscopic colitis. Immunomodulators seem to have low response rate 0%-56% for patients with refractory microscopic colitis. Response rate 66%-100% was observed for use of anti-tumor necrosis factor (TNF) therapy for refractory microscopic colitis. Anti-TNF and diverting ileostomy may be an option in severe or refractory microscopic colitis.

Key words: Chronic diarrhea; Etiology of microscopic colitis; Collagenous colitis; Refractory microscopic colitis; Microscopic colitis; Lymphocytic colitis; Steroid-dependent microscopic colitis

Core tip: The etiology of microscopic colitis (MC) is unknown. There is a strong association with autoimmune disorders, smoking, and medications,

such as non-steroidal anti-inflammatory drugs, proton pump inhibitors, and selective serotonin reuptake inhibitors. There are no societal guidelines on how to manage patients with MC. Data is strongest for the use of budesonide. Budesonide can rapidly induce clinical remission but relapse occurs frequently after discontinuation of budesonide. Anti-diarrheals may be used alone in mild MC or in conjunction with other therapies in moderate to severe MC. There is limited data on management of steroid-dependent or refractory MC but anti-TNF and diverting ileostomy may be options.

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INTRODUCTION

Microscopic colitis (MC) is a common cause of chronic, nonbloody diarrhea. Up to 10%-20% of chronic diarrhea is thought to be secondary to MC. The incidence of MC in Sweden, Spain, and Iceland from the 1990s ranged from 1.1 to 5.2 per 100000 person-years[1]. In a population based cohort study conducted from 1985 to 2001 in Olmsted County, Minnesota, the incidence of CC was 3.1 per 100000 and that of LC was 5.5 per 100000[1]. Poisson regression analysis showed that the incidence of MC increased over time, and the incidence of MC by the end of 2001 was 19.6 per 100000[1]. MC typically affects patients in their 50-60 s and occurs more frequently in women than men. The diagnosis is made by both clinical history and endoscopic biopsies. While chronic watery diarrhea is the most common symptom, some patients with MC may also experience abdominal pain, fecal incontinence, and/or weight loss. Colonoscopy generally reveals normal colonic mucosa but colonic biopsy shows classic histological features: > 20 intraepithelial lymphocytes per 100 epithelial cells in lymphocytic colitis (LC) and 10-20 μm of a thickened subepithelial collagen band in collagenous colitis (CC) (Figure 1). Inflammation in the lamina propria, with mainly mononuclear cells, may be seen in CC. Other etiologies, such as celiac disease, inflammatory bowel disease, and infectious colitis should be ruled out.

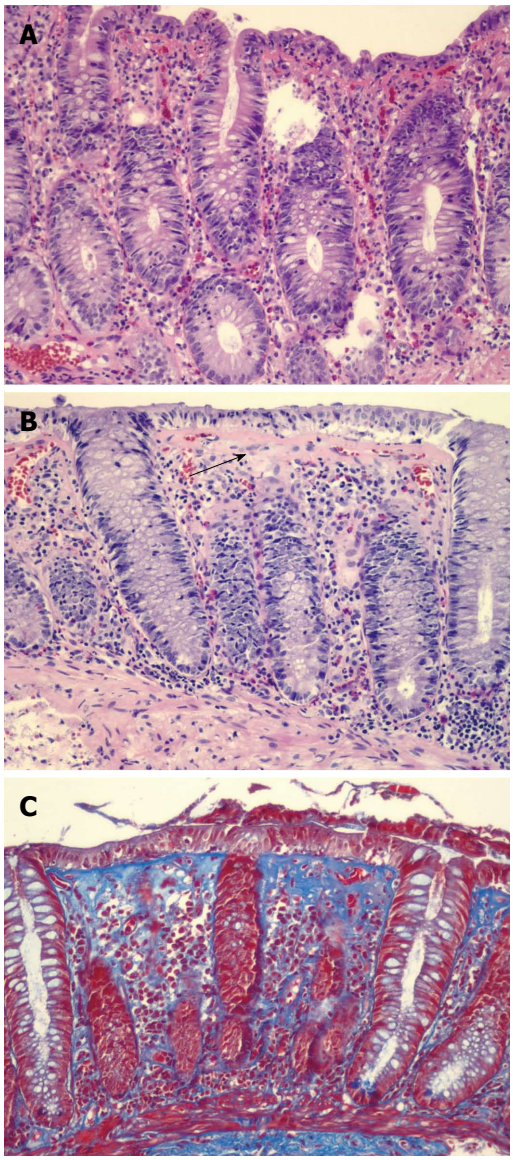

Figure 1 Colonic biopsy. A: Lymphocytic colitis, hematoxylin and eosin stain. The key histological feature is increased number of intraepithelial lymphocytes with little or no crypt architectural distortion. The presence of > 20 intraepithelial lymphocytes per 100 surface epithelial cells is diagnostic of lymphocytic colitis (LC)[2]. Fewer than 5 intraepithelial lymphocytes per 100 surface epithelial cells are considered normal[2]. Inflammatory infiltration usually consists of lymphocytes and plasma cells but eosinophils and neutrophils may also be present; B: Collagenous colitis, hematoxylin and eosin stain. The black arrow points to the thickened subepithelial collagen band. Band thickness is 10-20 μm in collagenous colitis (CC)[2]. Collagen band thickness < 3 μm is considered normal[2]; C: Collagenous colitis, trichrome stain. Collagen stains blue on trichrome stain. Trichrome stain can be useful when it is difficult to determine the thickness of collagen band on hematoxylin and eosin stain. This picture demonstrates a thickened subepithelial collagen band.

ETIOLOGY

The etiology of MC is unknown. There is a strong association with autoimmune disorders, such as celiac disease, polyarthritis, and thyroid disorders (Table 1). Up to twenty to 60% of patients with LC and 17%-40% of patients with CC have autoimmune disease[2]. In fact, histological features of MC in the colon are present in 30% of patients with celiac disease. While no specific genetic mutations have been identified as direct cause of MC, some studies have found common genetic abnormalities. For instance, there is an increased prevalence of human leukocyte antigen (HLA) DR3 DQ2 allele in patients with MC, and metalloproteinase 9 gene variations have been associated with CC[3].

Smoking is a risk factor for MC. In a prospective,

Table 1 Factors associated with microscopic colitis

Autoimmune disorder (type 1 diabetes, rheumatoid arthritis, celiac disease, thyroid disorders)
Nonsteroidal anti-inflammatory drugs
Proton pump inhibitors
Serotoin reuptake inhibitors
Statins
Beta-blockers
Smoking

case-control study conducted from 2007 to 2010 in Spain involving 255 patients, smoking was significantly associated with LC and CC (OR = 3.8 in LC, OR = 2.4 in CC)[4]. Swedish study conducted by Vigren *et al*[5] also showed that smoking is associated with CC. Thirty-seven percent of patients with CC were smokers as compared to only 17% of patients in the control group (OR = 2.95). Subgroup analysis showed that the association of smoking with CC was most notable in the age group 16-44; 75% of patients in this age group were smokers as compared to 15% in the control group (odd ratio: 16.54). Smokers develop MC earlier than nonsmokers by a median of 14 years[6].

Medications are often implicated as a cause of MC. Non-steroidal anti-inflammatory drugs (OR = 4.6 in LC, OR = 3.8 in CC), proton pump inhibitors (OR = 2.7 in LC, OR = 6.4 in CC), selective serotonin reuptake inhibitors (OR = 17.5 in LC), and beta-blockers (OR = 3.6 in CC) are strongly associated with MC (Table 1)[4]. A population-based case-control study using a Dutch primary care database assessed the risk of MC from exposure to medications compared with community control as well as with colonoscopy control where subjects had negative colonoscopy and negative histology for MC[7]. This study showed that current use of proton pump inhibitor (OR = 10.6) and nonsteroidal anti-inflammatory drugs (OR = 5.6) significantly increased the risk of MC[7]. Diclofenac was the most commonly used non-steroidal anti-inflammatory drug, and omeprazole was the most commonly used proton pump inhibitor in this study. Selective serotonin reuptake inhibitors, beta-blockers, and ACE inhibitors increased the risk of MC when compared with community controls, but not when compared with colonoscopy controls[7].

A more recent theory centers around bacterial translocation in the gastrointestinal tract. Bacterial antigens or toxins are suspected to increase inflammatory mediators in the colonic mucosa, leading to increased mucosal permeability, increased cytokines, degradation of the collagen matrix, and dysregulation of intestinal subepithelial myofibroblasts. No specific organisms have been identified in causing or exacerbating MC. Analysis of colonic biopsies of patients with MC demonstrated an increased amount of interferon-gamma, tumor necrosis factor alpha, and interleukin-1β in patients with MC as compared to patients without MC who underwent routine screening/surveillance colonoscopy, suggesting role of Th1 immune response in MC[8]. There was a trend towards increased level of interleukin-13 in patients with MC but interleukin-13 levels were not significantly different between MC and non-MC patients[8]. There was no difference in interleukin-8 level between the two groups[8]. Mucosal mRNA levels of interferon-gamma and interleukin-15 were 100 times greater and tumor necrosis factor alpha was 60 times greater in patients with MC as compared to patients with irritable bowel syndrome with diarrhea predominance, also supporting the role of Th1 immune response, which may be triggered by an unknown luminal antigen and ultimately leads to MC in susceptible individuals[9].

MANAGEMENT

There are no society guidelines on how to best manage patients with MC. While some provider will start with the most benign recommendations including lifestyle changes and work their way up to anti-diarrheals and steroids, others will go right to the most effective medications.

Lifestyle changes

As an initial approach, a thorough history should be taken in order to corroborate a temporal relationship between starting a new medication and the onset of symptoms. If suspected, offending medications should be discontinued if possible. Smoking cessation should also be encouraged.

Anti-diarrheal medications (loperamide, diphenoxylate/atropine)

There are no randomized controlled trials comparing efficacy of antidiarrheals against placebo or other medical therapy for MC. A retrospective registry review of 163 patients with CC showed a 71% efficacy rate (49 out of 69 patients) of loperamide[10]. Consensus is that anti-diarrheals may be used alone in mild MC or in conjunction with other therapies in moderate to severe MC to reduce frequency of diarrhea.

Bismuth

In an animal study, bismuth subsalicylate and bismuth subcitrate enemas significantly reduced the macroscopic and microscopic appearance of the 2, 4, 6-trinitrobenzene sulfonic acid (TNBS)-induced colitis in rats compared to TNBS-induced rats treated with saline enemas[11]. TNBS-induced colitis is a model for chronic inflammation and ulceration seen in inflammatory bowel disease rather than MC; therefore, this animal study does not directly demonstrate benefit of bismuth in MC. However, its effect on reduction of microscopic injury scores can be used to infer its benefit in MC. A prospective study of 12 patients with MC treated with bismuth subsalicylate 262 mg/d for

8 wk showed a 92% rate of clinical remission and a 75% rate of histological resolution of MC[12]. Mean time to response was 2 wk. At 7-28 mo follow-up, 75% of patients remained in remission.

Cholestyramine

There are no randomized controlled trials demonstrating efficacy of cholestyramine for MC. In a small retrospective study involving 27 patients with CC, 12 out of 27 (44%) patients were found to have abnormal bile acid absorption. Eleven out of these 12 patients (92%) reported improvement in diarrhea within one week when treated with bile acid binding agents (cholestyramine 4 g 2-3 times per day, or colestipol 5 g 2-3 times per day if the patients could not tolerate the smell or taste of cholestyramine)[13]. Ten out of 15 (67%) patients with CC and normal bile acid absorption reported improvement in diarrhea when treated with bile acid binding agents[13]. Overall, 78% of patients with CC experienced improvement in diarrhea with bile acid binding agents. In a retrospective registry review of patients with CC, cholestyramine was effective in 26 out of 44 patients (59%)[10].

Aminosalicylates (5-aminosalicylic acid)

In a study of 64 patients with MC treated with mesalamine 2.4 g/d *vs* mesalamine 2.4 g/d plus cholestyramine 4 g/d for 6 mo, the rates of remission were similar between the two groups. Eighty four percent of patients experienced clinical remission within the first 2 wk of treatment[14]. Clinical remission occurred earlier in patients treated with mesalamine plus cholestyramine than those treated with mesalamine alone.

Other studies have demonstrated little benefit of aminosalicylates. In a double-blinded, randomized placebo-controlled trial of 92 patients comparing efficacy of budesonide, mesalamine, and placebo, interim analysis found mesalamine to be less effective than placebo in inducing clinical remission and the review board recommended closure of this study arm[15]. The rate of clinical remission was 44% in mesalamine group and 59.5% in placebo group at 8 wk[15].

Corticosteroids

A number of medications have been studied to induce clinical remission, and the evidence is strongest for the use of budesonide. A prospective, double-blinded, randomized controlled trial comparing induction of budesonide (9 mg/d for 4 wk, 6 mg/d for 2 wk, alternating doses of 6 and 3 mg/d for 2 wk) followed by maintenance dose of budesonide (alternating 6 and 3 mg/d) *vs* placebo for 1 year showed that maintenance of clinical remission rate was higher in budesonide group (61.4%) than in the placebo group (16.7%)[16]. Clinical remission rate after induction period was 84.5% and the median time to remission was 10.5 d[16].

Similar randomized controlled trials comparing induction of budesonide followed by maintenance with budesonide *vs* placebo have shown high clinical remission rate 77%-96% in budesonide group (Table 2). A Cochrane review by Chande *et al*[17] showed that the pooled odds ratio: for inducing clinical response with budesonide was 12.32 (95%CI: 5.53-27.46) and for maintaining clinical response was 8.82 (95%CI: 3.19-24.37)[17]. The number needed to treat was 2 for each outcome. Additionally, patients treated with budesonide had a higher rate of complete response than those treated with prednisone (82.5% *vs* 52.9%; OR = 4.18; 95%CI: 1.3-13.5) and were less likely to recur than those treated with prednisone (HR = 0.38; 95%CI: 0.18-0.85; $P = 0.02$)[18].

A multicentered, randomized controlled trial of 92 patients comparing budesonide, mesalamine, and placebo for MC showed that budesonide was more effective than mesalamine (80% *vs* 44%, $P = 0.0035$) and placebo (80% *vs* 59.5%, $P = 0.072$) in inducing clinical remission at 8 wk[15]. Histological remission rate was the highest in patients treated with budesonide (87%) as compared to mesalamine (45%) and placebo (50%)[15]. The rates of adverse events were similar among budesonide, mesalamine, and placebo groups (47%, 68%, 54%)[15]. The most frequent adverse events were nasopharyngitis, headaches, and dyspepsia.

Although budesonide has been shown to rapidly induce clinical response, relapse occurs frequently after discontinuation of budesonide. Relapse rate is estimated to be as high as 26%-82% (Table 2). Median time to relapse after stopping active treatment was 39 d[19]. Patients with baseline diarrhea frequency > 5 per day (HR = 1.67), duration of diarrhea > 12 mo (HR = 1.82), and absence of budesonide maintenance therapy (HR = 2.73) were found to be at highest risk for relapse[20]. Other factors associated with relapse were advanced age ($P = 0.047$), a higher number of bowel movements per day at randomization after induction period ($P = 0.009$), and a higher number of bowel movements per day at baseline ($P = 0.041$)[16].

Immunomodulators

Immunomodulators, such as azathioprine, 6-mercaptopurine, and methotrexate, have been tried in patients with refractory MC or steroid dependent MC (Table 3). There are no randomized controlled trials studying efficacy of these medications for MC, and data on use of these medications for refractory MC is limited.

In a small study of 9 patients with CC who were intolerant or nonresponsive to budesonide, the patients were treated with methotrexate 15-25 mg/wk subcutaneously for 12 wk. Four of 9 patients discontinued methotrexate due to adverse effects (nausea, allergic reaction, worsening diarrhea), and none of the patients achieved clinical remission at

Table 2 Summary of studies demonstrating efficacy of budesonide for treating microscopic colitis

	Number of patients	Mean age (yr)	Treatment	Remission rate[1]	Relapse rate[2]
Miehlke *et al*[31], 2008	48	57.5	Budesonide 9 mg/d for 6 wk, followed by budesonide 6 mg/d *vs* placebo for 6 mo	Short-term 96% Long-term 74% *vs* 35% placebo, $P = 0.008$	26% *vs* 65% placebo, $P = 0.022$
Bonderup *et al*[19], 2009	34	62.8	Budesonide 9 mg/d for 6 wk, followed by budesonide 6 mg/d *vs* placebo for 24 wk	Long-term 76.5% *vs* 12% placebo, $P < 0.001$	53%
Miehlke *et al*[15], 2014	92	58.8	Budesonide 9 mg/d *vs* placebo for 8 wk	At 8 wk, 80% *vs* 59.5% placebo, $P = 0.072$	35%
Münch *et al*[16], 2014	92	56.7	Budesonide 9 mg/d for 8 wk, followed by budesonide 4.5 mg/d *vs* placebo for 6 mo	Short-term 84.5% Long-term 61.4% *vs* 16.7% placebo, $P < 0.001$	82.1% *vs* 12.5% placebo

[1]Short-term remission rate is within 6 wk during induction phase. Long-term remission rate is at 6 mo of maintenance therapy, unless otherwise specified; [2]After discontinuation of therapy.

Table 3 Response rate to immunomodulators and anti-tumor necrosis factor for severe or refractory microscopic colitis

	Number of patients	Mean age (yr)	Treatment	Duration of therapy	Response rate
Münch *et al*[22], 2013	46	59	Azathioprine or 6-MP 2 mg/kg per day	Variable, 1-57 mo	41%
Pardi *et al*[30], 2001	9	62	Azathioprine or 6-MP 2 mg/kg per day	26 mo	56%
Münch *et al*[21], 2013	9	62	Methotrexate 15-25 mg/wk	12 wk	0%
Esteve *et al*[25], 2011	4	59	Infliximab 5 mg/kg at 0, 2, 6 wk then every 6-8 wk intravenously	Variable, 5-14 mo	75%
Münch *et al*[26], 2012	3	55	Adalimumab induction[1] then 40 mg subcutaneously every 2 wk	6 wk	66%
Pola *et al*[24], 2013	1	58	Infliximab 5 mg/kg at 0, 2, 6 wk then every 8 wk intravenously	6 mo	100%

[1]Adalimumab induction: 160 mg (week 0), 80 mg (week 2), and 40 mg (week 4).

week 12[21].

A multicentered European study showed 41% overall response rate to thiopurines (azathioprine or 6-mercaptopurine) in patients who were steroid dependent or failed other medical therapy, such as loperamide, cholestyramine, budesonide, mesalamine, and methotrexate[22]. Nine patients with steroid intolerance, refractoriness, or dependence were treated with azathioprine 2 mg/kg per day and were followed for 26 mo. Eight out of 9 patients (89%) had complete (56%) or partial (33%) response to azathioprine and were able to discontinue corticosteroids. One out of 9 patients (11%) had persistent severe diarrhea and required an ileostomy.

Anti-tumor necrosis factor

There are no randomized controlled trials studying efficacy of anti-tumor necrosis factor (TNF) for refractory MC, but there are multiple case reports of clinical response after induction therapy with either infliximab or adalimumab (Table 3). Aram *et al*[23] reported a patient with lymphocytic enterocolitis who failed corticosteroids, antibiotics, cholestyramine, azathioprine, and tincture of opium. This patient was treated with infliximab 5 mg/kg at 0, 2 and 6 wk with resolution of diarrhea. There was a report of a patient with steroid-dependent MC who failed to respond to diphenoxylate/atropine, loperamide, bismuth, mesalamine, cholestyramine, octreotide, alosetron, tincture of opium, and *Boswellia serrata* extract was treated with infliximab 5 mg/kg and methotrexate 12.5 mg per week. After 6 mo, the patient remained asymptomatic and free of corticosteroids[24].

Several small case series also demonstrated improvement. Long-term remission (more than 1 year) was achieved in 3 out of 4 patients with refractory MC who were treated with anti-TNF[25]. One patient developed Stevens Johnson syndrome after starting infliximab and relapsed when switched to adalimumab; therefore, anti-TNF was discontinued. The patient underwent colectomy. In another series, three patients with MC who failed loperamide, cholestyramine, budesonide, and methotrexate were treated with adalimumab. All 3 patients achieved clinical remission at week 6 but 1 patient stopped therapy due to adverse effects (vomiting, abdominal pain)[26].

Diverting ileostomy

There are case reports of patients successfully undergoing colectomy or diverting ileostomy for refractory and severe MC. The observational trend from these case reports is that both symptoms and histology improve after surgery but can recur when bowel continuation is restored.

In one case report, a 33 year-old patient with 5 years of chronic diarrhea from CC not responsive to Asacol and prednisone underwent total protocolectomy followed by ileal pouch anal anastomosis. At 2 year follow-up, she was having multiple bowel movements per day but diarrhea resolved and she was able to return to full-time job[27].

In another, a 59 year-old patient with CC who previously failed loperamide, prednisolone, budesonide, 5-aminosalicylic acid, cholestyramine, and norfloxacin underwent loop ileostomy[28]. Two to 4 mo after loop ileostomy, colonic biopsies showed resolution of subepithelial damage, but her clinical course was complicated by *Clostridium difficile* infection and problems with the stoma. One year later, the patient improved clinically and loop ileostomy was closed. The patient started budesonide 6mg daily after bowel restoration but relapsed with symptoms of CC.

In a series of patients, nine patients with MC refractory to medical therapy (sulfasalazine, mepacrine, corticosteroids, mesalamine, cholestyramine, loperamide, metronidazole) underwent ileostomy in between 1981-1992[29]. All patients experienced clinical and histological remission. The ileostomy was taken down in 5 patients after a diversion period of 4-15 mo. Diarrhea recurred in 4 out of 5 of these patients and 3 of them underwent additional surgery.

CONCLUSION

MC is characterized by chronic, watery diarrhea and subepithelial changes. Conservative therapy, such as discontinuation of offending mediations and trial of loperamide and/or bismuth, may be successful in treating non-severe MC. Budesonide can rapidly induce clinical remission and can maintain remission, but relapse occurs frequently after withdrawal of therapy. Methotrexate and mesalamine do not appear to be effective as monotherapy for MC. There is limited data to support the use of immunomodulators in refractory or steroid-dependent MC. Anti-TNF or ileostomy may be an option for severe, refractory MC; however, their efficacy has not been proven in randomized controlled trials yet, and their risk and benefits need to be discussed in detail with the patient prior to recommending these therapy.

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MINIREVIEWS

Chemotherapy beyond second-line in advanced gastric cancer

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Abstract

Patients with advanced gastric cancer (AGC) can be treated with multiple lines of chemotherapy. Although several randomized trials have demonstrated the benefit of second-line chemotherapy compared with best supportive care, there is no evidence that further lines of chemotherapy will result in substantial prolongation of survival. Despite this, the practice of offering chemotherapy beyond second-line agents to AGC patients is not uncommon if their performance status is well-preserved and they are willing to receive subsequent active treatments. The choice of chemotherapeutic agents depends on the patient's prior regimens. However, there are important controversial issues in the salvage setting of AGC, including a subset of patients who may benefit from chemotherapy, that still remain unanswered. This report reviews the available evidence regarding the impact of third- and subsequent lines of chemotherapy on survival and quality of life in patients with AGC.

Key words: Chemotherapy; Gastric cancer; Salvage

Core tip: There no evidence to date that chemotherapy beyond second-line has a beneficial effect in patients with gastric cancer. The impact of third- and subsequent lines of chemotherapy on survival and quality of life is the subject of this review.

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INTRODUCTION

Although fluoropyrimidines and platinum combination chemotherapy is considered standard first-line treatment in patients with advanced gastric cancer (AGC)[1], the overall prognosis of such patients remains

Table 1 Randomized phase Ⅲ trials in the second-line treatment of gastric cancer

Trials	Treatment	No. of patients	OS (mo)	HR for OS (95%CI)	Remarks
AIO[8]	Irinotecan	21	4.0	0.48 (0.25-0.92)	Closed early due to poor accrual
	BSC	19	2.4	P = 0.012	
Korean[9]	Docetaxel or irinotecan	133	5.3	0.657 (0.485-0.891)	No OS difference between docetaxel
	BSC	69	3.8	P = 0.007	(5.5 mo) and irinotecan (6.5 mo)
COUGAR-02[10]	Docetaxel	84	5.2	0.67 (0.49-0.92)	Global QOL similar between arms
	BSC	84	3.6	P = 0.01	(P = 0.53)
REGARD[11]	Ramucirumab	238	5.2	0.776 (0.603-0.998)	
	Placebo	117	3.8	P = 0.047	
RAINBOW[12]	Ramucirumab + paclitaxel	330	9.6	0.807 (0.678-0.962)	
	Placebo + paclitaxel	335	7.4	P = 0.017	
WJOG 4007[15]	Paclitaxel	108	9.5	1.14 (0.88-1.49)	
	Irinotecan	111	8.4	P = 0.24	

BSC: Best supportive care; QOL: Quality of life.

poor[2]. Clinical trials involving novel, targeted agents have had little success as first-line treatment for AGC, with the exception of trastuzumab in patients with human epidermal growth factor receptor 2 (HER2)-positive tumors[3]. Fortunately, in a second-line setting, we now have evidence that chemotherapy provides substantial improvement in overall survival (OS) and quality of life (QOL)[4]. While it is common practice to offer second- or further lines of chemotherapy to AGC patients[5], and more than a few randomized trials have shown improved OS for certain second-line regimens[4], chemotherapy beyond second-line is associated with responses in fewer patients[6] and has no clinical relevant or consistent effects on OS. Since patients with progressive disease usually have poor performance status, aggressive chemotherapy may not be feasible. It is also known that response rates to chemotherapy decline with each subsequent regimen. Nevertheless, many AGC patients still receive chemotherapy beyond second-line, and in some cases are treated until death occurs, since patients and physicians sometimes have difficulty with transitioning to only supportive care. Considering the grim prognosis for AGC patients with second-line failure, the value of third- and subsequent lines of chemotherapy may be better evaluated by other outcome measures, such as improvement in QOL. This article reviews the available evidence regarding the impact of third- and subsequent lines of chemotherapy on OS and QOL in patients with AGC.

SECOND-LINE THERAPY

Even before the role of second-line chemotherapy was recently described, second- or further lines of chemotherapy have been administered for AGC patients after first-line failure[7]. Our own retrospective analysis of AGC patients who received second-line chemotherapy found a median survival of 6.7 mo[5], with baseline hemoglobin level and performance status being independent prognostic factors. There is a general consensus that the role of second- and subsequent lines of chemotherapy, also called "salvage" therapy, in prolonging OS in AGC patients is modest. Response rates are less than 20% and short-lived, with a median OS of 4-6 mo[4]. The results of several phase Ⅲ trials testing the role of second-line therapy in patients with AGC have been reported (Table 1)[8-12]. In this setting, three cytotoxic chemotherapeutic agents (paclitaxel, docetaxel, and irinotecan) and one anti-vascular endothelial growth factor receptor (anti-VEGFR) antibody (ramucirumab) have shown significant reductions in the risk of death. Notably, the hazard ratios (HRs) in all of these single-agent trials were of a similar magnitude, which is indicative of the robustness of the findings, since the investigators assessed the treatments in patients of different ethnic origins[4]. The first trial conducted by German Arbeitsgemeinschaft Internistische Onkologie (AIO) investigators included 40 patients who received either irinotecan or best supportive care (BSC)[8], and showed significant benefit with second-line irinotecan compared with BSC alone. The second trial was conducted by the current authors[9], in which second-line chemotherapy with either docetaxel or irinotecan was compared with BSC in 202 Korean patients. We reported a significant survival benefit with second-line chemotherapy (5.3 mo *vs* 3.8 mo, HR = 0.657, 95%CI: 0.485-0.891). In the third, a COUGAR-02 trial[10], researchers from the United Kingdom reported that second-line docetaxel improved median OS compared with BSC (5.2 mo *vs* 3.6 mo, HR = 0.67, 95%CI: 0.49-0.92). The trial included QOL as one of the objectives and reported similar global health-related QOL scores between the chemotherapy and BSC arms.

Despite the failure of more than a few clinical trials involving targeted agents[13,14], two phase Ⅲ trials of ramucirumab[11,12], either as monotherapy or in combination with paclitaxel, were successful. In the REGARD trial, pretreated AGC patients were randomly assigned to receive ramucirumab or a placebo as

second-line treatment. Surprisingly, the survival benefit achieved with ramucirumab (5.2 mo *vs* 3.8 mo, HR = 0.776, 95%CI: 0.603-0.998) was similar to that seen in phase Ⅲ trials. In the RAINBOW trial[12], a more clinically relevant trial that is the largest to date, the addition of ramucirumab to paclitaxel was compared to paclitaxel alone for second-line therapy. The authors reported that OS was significantly longer in the ramucirumab plus paclitaxel arm than in the paclitaxel monotherapy arm (9.6 mo *vs* 7.4 mo, HR = 0.807, 95%CI: 0.678-0.962).

However, which regimen should be the standard of care in the second-line setting still remains unclear. For patients who failed fluoropyrimidine and platinum, paclitaxel[12,15], docetaxel[9,10], and irinotecan[9,15] have all been evaluated extensively in clinical trials. Combination chemotherapy may achieve higher response rates than monotherapy, but the survival outcomes are the same[16]. In our own retrospective analysis performed in 1455 AGC patients[5], there was no relevant difference in median OS between patients who were treated with second-line combination and monotherapy. In addition, to achieve palliative goals with second-line chemotherapy, patients are more likely to tolerate single agents than combination therapy. Hironaka *et al*[15] reported the results of a phase Ⅲ trial comparing irinotecan with paclitaxel in the second-line setting, and found that OS was not significantly different (9.5 mo in paclitaxel arm *vs* 8.4 mo in irinotecan arm, HR = 1.13, 95%CI: 0.86-1.49). Clearly, medically fit patients who failed or were refractory to first-line chemotherapy should receive second-line chemotherapy, with BSC reserved for those with a poor performance status. It should be noted that AGC is a heterogeneous disease, with substantial differences in its aggressiveness and responsiveness to therapy. The clinical outcome and prognosis in individual patients do not always conform to the published data. In daily clinical practice, outside of the strict enrollment criteria of a clinical trial, many AGC patients develop peritoneal carcinomatosis during the course of their disease[5], leading to rapid symptomatic deterioration and chemotherapy intolerance.

THIRD-LINE THERAPY

It seems clear that, for the majority of patients, the benefit of chemotherapy beyond second-line for advanced disease is minimal to modest. However, as described above, some AGC patients still are candidates for third- or subsequent lines of therapy, despite not having an established third-line regimen to offer. More than two-thirds of patients enrolled in the Japanese second-line chemotherapy trial were treated with third-line therapy[15]. In our own Korean phase Ⅲ trial[9], 27% of patients had received study treatment as third-line therapy, with the survival benefit of chemotherapy being preserved (HR = 0.812, 95%CI: 0.450-1.464). Nevertheless, data from these phase Ⅲ trials should be interpreted carefully because of the potential selection bias; only a small percentage of patients continue to have good performance status after second-line therapy and they are still medically fit to be offered further therapy.

CYTOTOXIC CHEMOTHERAPY

The data on third-line chemotherapy are not conclusive, since published studies have included only a small number of patients within different patient subsets. The majority of published studies have been small phase Ⅱ or retrospective studies that have evaluated the feasibility of monotherapy or combinations of several cytotoxic agents. Because most AGC patients are initially treated with fluoropyrimidines and platinum[17], it is not a good idea to include these drugs in a salvage regimen for these patients. In the third-line setting, based on the lack of cross-resistance between taxanes and irinotecan, these chemotherapeutic agents are still plausible salvage treatment options[18]. Due to the risk of severe myelosuppression that is associated with the administration of paclitaxel or docetaxel every 3 wks, taxane monotherapy was commonly used as a weekly regimen for patients with heavily-treated disease. In small phase Ⅱ studies involving paclitaxel[19] or docetaxel[18,20], response rates were in the range of 15%-23%, with a median OS of 4-7 mo. Irinotecan is another commonly-used chemotherapeutic agent in AGC, with a similar single-agent efficacy to taxanes[9]. However, we should keep in mind that response rates and progression-free survival (PFS) do not always translate into a survival benefit. The choice of a third-line regimen should depend on previous treatments and, needless to say, on the patient's general condition. It is possible that the OS achieved in AGC was strongly associated with patient access to the three active chemotherapy regimens during the whole treatment course (*i.e.*, fluoropyrimidine/platinum-based first line, and second- and third-line chemotherapy with taxanes and irinotecan), which is similar to a model developed in patients with colorectal cancer[21].

NOVEL TARGETED THERAPY

When we consider the decline in patients' performance status and tolerability to cytotoxic chemotherapy, especially after failure of second-line therapy, more effective but less toxic treatment options are needed to provide an OS benefit for patients with AGC. In the first-line setting, the HER2-directed monoclonal antibody trastuzumab was shown to be effective in HER2-positive AGC[3]. However, trials involving another HER2 inhibitor, lapatinib, failed to show an OS benefit in the second-line setting[14].

Targeting angiogenesis *via* the inhibition of VEGFR has been another promising strategy in AGC. Although the Avastin in Gastric Cancer (AVAGAST) trial failed to

show a significant OS benefit (12.1 mo *vs* 10.1 mo, HR = 0.87, 95%CI: 0.73-1.03)[22], adding bevacizumab to first-line capecitabine and cisplatin chemotherapy was associated with increases in PFS and response rates. One may argue that this lack of correlation between the OS and PFS may be due to the lack of statistical power necessary to detect modest survival gain. We now have more optimistic results from the REGARD[11] and RAINBOW[12] trials involving ramucirumab in the second-line setting of AGC, as described above.

Similarly, small molecule inhibitors targeting VEGFR have been investigated in patients with AGC. However, the efficacy seen in phase Ⅱ studies with sunitinib as a potential second-line treatment for AGC patients has been modest[23,24]. We reported a prospective randomized trial comparing second-line docetaxel monotherapy with docetaxel plus sunitinib[24], in which the addition of sunitinib to docetaxel did not prolong PFS. One of the most promising VEGFR inhibitors at present is apatinib. A randomized, placebo-controlled phase Ⅱ trial conducted by Chinese investigators showed that apatinib improved PFS and OS in heavily-treated AGC patients[25]. Of note, 43% of patients given apatinib as third-line therapy achieved disease control, which justified further testing in a phase Ⅲ trial.

At the Annual Meeting of the American Society of Clinical Oncology (ASCO) in June 2014, Qin *et al*[26] presented a randomized phase Ⅲ trial comparing apatinib with a placebo in 273 AGC patients with prior failure to second-line chemotherapy. The primary endpoint was OS and the secondary endpoints were response rate, PFS, safety, and QOL. The apatinib arm had superior PFS (78 d *vs* 53 d, HR = 0.44, 95%CI: 0.33-0.61), response rate (3% *vs* 0%), and median OS (195 d *vs* 140 d, HR = 0.71, 95%CI: 0.54-0.94) compared to placebo, with a manageable safety profile. Patients receiving apatinib had a higher incidence of neutropenia and thrombocytopenia, as well as proteinuria and hypertension. Additionally, severe (grade 3 or 4) hand-foot syndrome occurred in 8.5% of patients in the apatinib arm. As differences regarding QOL were not included in the presentation, full publication of this study will be of interest.

SELECTION OF PATIENTS

Since patients' QOL and performance status would diminish with advanced lines of chemotherapy, the expected OS benefit, if any, is the single most important factor in choosing a salvage regimen for AGC. To the best of our knowledge, no prospective analyses have been performed to examine prognostic and/or predictive factors in the third-line setting. Nevertheless, it is known that some patient and tumor factors are particularly helpful in selecting patients who may benefit from salvage therapy, and should be evaluated carefully. Among the clinical and laboratory factors, the most important are the patient's performance status, some hematological and laboratory values (including hemoglobin level or serum albumin), the number of previous lines of therapy and the response obtained, and the number and site of metastases[5,27]. In our retrospective study[5], for patients who received supportive care only in a salvage setting, the reasons for such a decision included poor performance status (71%) and the patient's refusal (29%). Known tumor characteristics that directly influence the aggressiveness of the disease, such as peritoneal carcinomatosis, tumor grade, and Lauren classification, are also helpful. In addition, research is under way to define specific predictive factors of responsiveness to certain types of therapy. The Cancer Genome Atlas (TCGA) research network, for example, reported a comprehensive molecular evaluation of 295 gastric adenocarcinomas[28] and proposed four distinct molecular subtypes: (1) tumors positive for Epstein-Barr virus; (2) microsatellite unstable tumors; (3) genomically stable tumors; and (4) tumors with chromosomal instability. Experience with other types of cancer has taught us that a substantial improvement in the treatment of AGC could be achieved with individualized therapy strategies[29], including the identification of genetic alterations and the study of the molecular biology of therapeutic agents.

CONCLUSION

Although the current evidence is lacking concerning potential beneficial effects associated with administering third- or subsequent lines of chemotherapy, it is common practice to offer further chemotherapy for AGC patients after second-line failure[5]. In this setting, no chemotherapeutic agents or regimens have a proven survival benefit over supportive care only, and thus no standard salvage therapy exists. Although taxanes and irinotecan have shown efficacy in this setting, no randomized trials have been conducted, and these regimens have low response rates. Recently, a phase Ⅲ trial conducted in China demonstrated a benefit with apatinib in this setting[26], which is a novel, orally-administered VEGFR inhibitor. It is therefore very important to emphasize that all treatment decisions must be individualized; targeting the specific histological and biological features that make a tumor unique, and the clinical features that make a patient unique.

Evidence showing an OS benefit of therapy in third- or subsequent lines of chemotherapy in patients with AGC suggests that salvage therapy may indeed become the standard of care. Administration of an active and tolerable therapy regimen may have a beneficial effect on patients' QOL, as a direct result of improvements in clinical outcome. However, these studies are few in number and await further confirmation. Based on these considerations, giving a patient the opportunity to actively participate in the selection of treatment seems to be an important factor for patient satisfaction and improved QOL. Even in

heavily-treated AGC patients, salvage therapy may be of value in terms of QOL[30]. Furthermore, patient preference for treatment is increasingly important in clinical decision-making, and has been the subject of medical research[31,32]. We should acknowledge that while curative treatment is not currently available, different treatment strategies, including no active therapy, may be appropriate. Accordingly, we must be willing to take the time to accurately and extensively discuss all treatment options in order to select the best treatment for each particular patient.

In summary, the role of therapy beyond second-line in AGC has not yet been established. Despite recent advances, the prognosis of AGC patients remains poor. However, we have considerable indirect evidence from a number of phase Ⅱ or retrospective studies suggesting improved response rates and prolonged PFS through the use of third- or subsequent lines of chemotherapy. One may consider currently available chemotherapy regimens (*i.e.*, fluoropyrimidine plus platinum, taxanes, and irinotecan) for use during the whole treatment course, similar to that described for colorectal cancer[21], in which three active drugs (fluoropyrimidines, oxaliplatin, and irinotecan) should all be used. Recently, a prospective phase Ⅲ trial performed in Chinese AGC patients reported a survival benefit with the use of a novel, oral-targeted agent, apatinib. It is conceivable that integration of targeted agents, including ramucirumab and/or apatinib, into the treatment regimen could improve treatment efficacy in patients with AGC. While there is still controversy over the benefit of salvage therapy in the third-line setting and beyond, there should be certain patients who would derive the most benefit from the therapy. Our clinical expertise, better understanding of gastric carcinogenesis, and molecular characterization of this cancer will provide hope for more successful treatment in the future.

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ORIGINAL ARTICLE

Basic Study

Expression of renal Oat5 and NaDC1 transporters in rats with acute biliary obstruction

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Author contributions: Brandoni A has designed research, performed research, contributed new reagents, analyzed data, wrote the paper; Torres AM has designed research, performed research, contributed new reagents, analyzed data, wrote the paper.

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Abstract

AIM: To examine renal expression of organic anion transporter 5 (Oat5) and sodium-dicarboxylate co-transporter 1 (NaDC1), and excretion of citrate in rats with acute extrahepatic cholestasis.

METHODS: Obstructive jaundice was induced in rats by double ligation and division of the common bile duct (BDL group). Controls underwent sham operation that consisted of exposure, but not ligation, of the common bile duct (Sham group). Studies were performed 21 h after surgery. During this period, animals were maintained in metabolic cages in order to collect urine. The urinary volume was determined by gravimetry. The day of the experiment, blood samples were withdrawn and used to measure total and direct bilirubin as indicative parameters of hepatic function. Serum and urine samples were used for biochemical determinations. Immunoblotting for Oat5 and NaDC1 were performed in renal homogenates and brush border membranes from Sham and BDL rats. Immunohistochemistry studies were performed in kidneys from both experimental groups. Total RNA was extracted from rat renal tissue in order to perform reverse transcription polymerase chain reaction. Another set of experimental animals were used to

evaluate medullar renal blood flow (mRBF) using fluorescent microspheres.

RESULTS: Total and direct bilirubin levels were significantly higher in BDL animals, attesting to the adequacy of biliary obstruction. An important increase in mRBF was determined in BDL group (Sham: 0.53 ± 0.12 mL/min per 100 g body weight *vs* BDL: 1.58 ± 0.24 mL/min per 100 g body weight, $P < 0.05$). An increase in the urinary volume was observed in BDL animals. An important decrease in urinary levels of citrate was seen in BDL group. Besides, a decrease in urinary citrate excretion (Sham: 0.53 ± 0.11 g/g creatinine *vs* BDL: 0.07 ± 0.02 g/g creatinine, $P < 0.05$) and an increase in urinary excretion of H^+ (Sham: 0.082 ± 0.03 μmol/g creatinine *vs* BDL: 0.21 ± 0.04 μmol/g creatinine, $P < 0.05$) were observed in BDL animals. We found upregulations of both proteins Oat5 and NaDC1 in brush border membranes where they are functional. Immunohistochemistry technique corroborated these results for both proteins. No modifications were observed in Oat5 mRNA and in NaDC1 mRNA levels in kidney from BDL group as compared with Sham ones.

CONCLUSION: Citrate excretion is decreased in BDL rats, at least in part, because of the higher NaDC1 expression. Using the outward gradient of citrate generated by NaDC1, Oat5 can reabsorb/eliminate different organic anions of pathophysiological importance.

Key words: Cholestasis; Kidney; Transporters; Organic anions

Core tip: Organic anion transporter 5 (Oat5) is an organic anion/dicarboxylate exchanger which has impact on renal excretion of hormones, drugs and xenobiotics. The primary function of sodium-dicarboxylate cotransporter 1 (NaDC1) is to reabsorb filtered Krebs cycle intermediates, such as citrate. We found upregulations of both transporters and a decrease in urinary citrate excretion in bile duct-ligated rats. Citrate excretion is decreased at least in part, because of the higher NaDC1 expression. Using the outward gradient of citrate generated by NaDC1, Oat5 can reabsorb/eliminate different organic anions of pathophysiological importance. Attention might be paid for those drugs transported by this protein because their pharmacokinetics may be altered during cholestasis.

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INTRODUCTION

Kidneys perform an essential function in the removal and reabsorption of organic anions from circulation[1]. Several transport proteins are implicated in the renal tubular secretion and reabsorption of endogenous and exogenous compounds[2].

Organic anion transporter 5 [(Oat5), Slc22a19] has been characterized as an organic anion/dicarboxylate exchanger[2-5]. This protein is localized in the brush border membrane of proximal tubule straight segment (S3)[4,5]. Oat5 has been reported to interact with many anionic drugs, such as bumetanide, furosemide, penicillin G and non-steroidal anti-inflammatory drugs[3-5]. The function of the organic anion exchanger Oat5 in renal cells under pathological conditions has not been fully elucidated yet.

The sodium-dicarboxylate cotransporter 1 [NaDC1), Slc13a2] is located in the apical membrane of the S1, S2, and S3 segments of proximal renal tubule[6,7]. The primary function of this transporter is to reabsorb filtered Krebs cycle intermediates[7,8]. These compounds, such as succinate, citrate and α-ketoglutarate are important substrates for renal metabolism because they account for 10%-15% of oxidative metabolism in the kidney[8,9]. Furthermore, Krebs cycle intermediates are involved in the maintenance of the outward dicarboxylate gradient that is crucial for the normal function of Oat exchanger proteins at both membrane domains, apical (such as Oat5) and basolateral (such as Oat1 and Oat3)[1,2].

Jaundice in chronic bile duct-ligated rats has been associated with functional and metabolic disturbances of the kidney[10]. Altered absorption, distribution and elimination of drugs have been described in this pathology[11,12].

The purpose of the current study was to examine the effects of acute extrahepatic cholestasis on the expression of Oat5 and NaDC1 in rats, and the contribution of these effects on renal excretion of citrate.

MATERIALS AND METHODS

Experimental animals

Male Wistar rats (110-130 d) were used throughout the study. The animal protocol was designed to minimize pain or discomfort to the animals. Animals were cared for in accordance with the principles and guidelines for the care and use of laboratory animals, recommended by the National Academy of Sciences and published by the National Institute of Health (NIH publication 7th edition revised 1996) and recommended by regulations of the local ethics committee. All experimental procedures were approved by the Faculty of Biochemical and Pharmaceutical Sciences Institutional Animal Care and Use Committee (Res. No.

637/2012).

Surgical procedure of bile duct ligation (BDL group) was performed as previously described[13-15]. A parallel group of Sham rats was processed. Studies were performed 21 h after surgery. During this period, animals were maintained in metabolic cages in order to collect urine. The urinary volume was determined by gravimetry.

Biochemical determinations

Animals were anaesthetized with sodium thiopental (70 mg/kg body weight, ip). Blood was withdrawn by cardiac puncture from Sham and bile duct (BDL) animals. These samples were used to measure total and direct bilirubin, and creatinine serum levels.

The urine samples were used to determine the urinary levels of citrate, creatinine, glucose and proteins, alkaline phosphatase activity and pH.

Creatinine clearance was calculated employing the following formula: $[Creatinine]_{urine} \times$ Urine flow/ $[Creatinine]_{plasma}$

Biochemical analyses were performed with optimized spectrophotometric techniques, employing commercial kits (Wiener Laboratory, Rosario, Argentina) except for citrate measurements that were performed using a citric acid enzymatic kit (Boehringer Mannheim/R-Biopharm, Darmstadt, Germany).

Preparation of brush border membrane (BBM) from kidney:

BBM from Sham (n = 4) and BDL (n = 4) rats were isolated from kidneys by Mg/EGTA precipitation as previously described[13]. For each experimental group, four different preparations were made. Protein quantification of samples was performed using the method of Sedmak and Grossberg[16].

Electrophoresis and immunoblotting

Immunoblotting for Oat5 and NaDC1 were performed in renal homogenates (20 μg of protein) and BBM (10 μg of protein) as previously described[13-15]. The membranes were incubated overnight at 4 ℃ with a non-commercial rabbit polyclonal antibodies against rat Oat5 (at a dilution of 1:800) or against rat NaDC1 (at a dilution of 1:800) or a commercial mouse monoclonal antibody against human β-actin (at a dilution of 1:800). Specificity of Oat5 and NaDC1 antibodies has been described elsewhere[4,6]. Blots were processed for detection using a commercial kit (ECL enhanced chemiluminescence system, Amersham, Buckinghamshire, United Kingdom). To verify equal protein loading and transfer between lanes, Ponceau Red and antibody against human β-actin were used as previously reported[13-15,17]. The abundance of Oat5 and NaDC1 were normalized to β actin. The relative protein expression for Oat5 or NaDC1 was expressed as percentage, considering the mean Sham value as the 100%.

Immunohistochemistry studies

The immunohistochemistry studies were performed as previously described[13,15,18,19]. Kidneys from the different experimental groups were briefly perfused with saline, followed by perfusion with periodate-lysine-paraformaldehyde solution (0.0375 M phosphate buffer (pH 6.2) containing 0.01 M $NaIO_4$, 0.075 M lysine, 2% paraformaldehyde), through a cannula inserted in the abdominal aorta. The kidney slices were immersed in periodate-lysine-paraformaldehyde solution at 4 ℃ overnight. After that, the tissue was embedded in paraffin and paraffin sections were cut.

After deparaffining, some sections were used for routine haematoxylin-eosin staining, while others were incubated with 3% H_2O_2 for 15 min (in order to eliminate endogenous peroxidase activity) to perform Oat5 and NaDC1 renal immunohistochemistry. Then, the sections were incubated with blocking serum for 30 min and after that with non-commercial rabbit polyclonal antibody against rat Oat5 (diluted 1:100[19]) or against rat NaDC1 (diluted 1:500[19]) overnight at 4 ℃. The sections were rinsed with Tris-Buffered Saline containing 1% Tween (TBST). Right after, the sections were incubated with horseradish peroxidase (HPR) conjugated secondary antibody against rabbit immunoglobulin for 1 h. So as to detect HPR labelling, a peroxidase substrate solution with diaminobenzidine (0.05% diaminobenzidine in TBST with 0.05% H_2O_2) was used. The sections were counterstained with hematoxylin before being examined under a light microscope. Controls using preimmune serum, antiserum absorbed with excess synthetic peptide, or omission of primary or secondary antibody revealed no labelling.

RNA isolation and reverse transcription polymerase chain reaction

As previously described by Bulacio *et al*[20], total RNA was extracted from rat renal tissue using Trizol reagent (Invitrogen, Carlsbad, CA, United States) following the manufacturer's instructions. Samples were stored at -80 ℃ until used.

The cDNA was synthesized using SuperScript™ First-Strand Synthesis System for reverse-transcriptase polymerase chain reaction (RT-PCR) (Invitrogen, Carlsbad, CA, United States) according to manufacturer's instructions. cDNA samples were kept at -20 ℃ until assayed.

RT-PCR for Oat5, NaDC1 and 18S rRNA as housekeeping gene was performed on cDNA samples using the BOECO TC-SQ Thermal Cycler (Boeckel Co., GmbH Co., KG, Hamburg, Germany). Reaction conditions used for the PCR were: initial denaturation for 2 min at 94 ℃, denaturation for 15 s at 94 ℃, annealing for 30 s at 55 ℃ and elongation for 60 s at 72 ℃. The final elongation step was 72 ℃ for 10 min. The specific PCR primers used were: for Oat5: 5'-GGAGGCAGCAGAGACAAAAC-3' (forward) and

Table 1 Plasma total and direct bilirubin levels of Sham and BDL rats

	Sham (n = 4)	BDL (n = 4)
Total bilirubin (mg/L)	5.1 ± 0.4	43.6 ± 2.8[a]
Direct bilirubin (mg/L)	2.2 ± 0.3	34.4 ± 3.3[a]

Results are expressed as means ± SE. [a]$P < 0.05$ *vs* Sham.

Table 2 Urine flow, creatinine clearance, urinary levels of glucose and protein, alkaline phosphatase urinary activity, urinary citrate concentration, urinary citrate excretion and urinary proton excretion of Sham and BDL rats

	Sham (n = 4)	BDL (n = 4)
Urine flow (μL/min per 100 g bw)	2.10 ± 0.24	3.87 ± 0.57[a]
Creatinine clearance (mL/min per 100 g bw)	0.65 ± 0.03	0.53 ± 0.06
Glucose (g/g creatinine)	0.17 ± 0.10	0.17 ± 0.07
Protein (g/g creatinine)	1.15 ± 0.08	1.15 ± 0.10
Alkaline phosphatase (U/g creatinine)	266 ± 14	253 ± 20
Citrate concentration (g/L)	0.71 ± 0.16	0.07 ± 0.03[a]
Citrate excretion (g/g creatinine)	0.53 ± 0.11	0.07 ± 0.02[a]
Proton excretion (μmol/g creatinine)	0.082 ± 0.03	0.21 ± 0.04[a]

Results are expressed as means ± SE. [a]$P < 0.05$ *vs* Sham. bw: Body weight.

5′-TTGCTCCTCCTAATGATGCC-3′ (reverse), for NaDC1: 5′-GAACGATAAGATGCCCTGGA-3′ (forward) and 5′-TGAAGACAGATGGCTTGTGC-3′ (reverse), and for 18S rRNA: 5′-CGCGGTTCTATTTTGTTGGT-3′ (forward) and 5′-AGTCGGCATCGTTTATGGTC-3′ (reverse).

RT-PCR products were then resolved by electrophoresis in a 1.2% agarose gel stained with SYBR Safe™ and visualized using the Safe Imager™ blue light transilluminator. For semiquantitative measurement, images of the gels were acquired and quantification of the optical density (OD) of the bands was performed. The Oat5/18S rRNA or NaDC1/18S rRNA product ratio was calculated and used as an index of Oat5 or NaDC1 mRNA expression, respectively. The relative mRNA expression for Oat5 or NaDC1 was expressed as percentage, considering the mean Sham value as the 100%.

Medullar renal blood flow determination

Another set of experimental animals (Sham, n = 4; BDL, n = 4) were used to evaluate Medullar renal blood flow (mRBF) using fluorescent microspheres as previously described[14,21-23]. The mRBF were calculated by the formula: renal flow (mL/min) = fl/flref × R (mL/min), where fl is the fluorescence of renal tissue, flref is the fluorescence of reference blood flow sample, and R is the withdrawal rate of reference blood flow sample.

Materials

Chemicals were purchased from Sigma (St. Louis, Missouri, United States) and were analytical grade pure.

Statistical analysis

Results are expressed as mean ± SE. Statistical analysis was performed using an unpaired t-test. When variances were not homogeneous a Welch's correction was employed. P values less to 0.05 were considered significant. For these analyses GraphPad software was used. The statistical review of the study was performed by a biomedical statistician.

RESULTS

Total and direct bilirubin levels were significantly higher in BDL animals, attesting to the adequacy of biliary obstruction (Table 1).

An important increase in mRBF was determined in BDL group (mL/min per 100 g body weight; Sham, n = 4: 0.53 ± 0.12; BDL, n = 4: 1.58 ± 0.24, $P < 0.05$).

Table 2 shows an increase in the urinary flow of BDL animals. There were no significant differences between groups in creatinine clearance, glucose and protein urinary excretion, and the activity of alkaline phosphatase. An important decrease in urinary levels of citrate was seen in BDL group. Besides, a decrease in urinary citrate excretion and an increase in urinary excretion of H^+ were observed in BDL animals.

Figure 1 shows a significant increase in Oat5 protein expression in homogenates as well as in BBM in BDL group. Oat5 renal expression was also assessed by immunohistochemistry technique. As it is shown in Figure 2, strong Oat5 labelling was associated with the apical membrane domains in proximal tubule cells. Oat5 staining of proximal tubule cells was increased in BDL group, consistent with the density observed by Western blotting studies in each experimental group.

Figure 3 shows a higher abundance of NaDC1 in BBM from BDL rats while no difference was observed in NaDC1 protein expression in homogenates. Immunohistochemistry showed labelling of NaDC1 associated with apical plasma membranes of proximal tubule of Sham and BDL rat kidneys (Figure 4). BDL rats showed an increased apical NaDC1 expression, corroborating the data obtained by Western blotting.

Oat5 and *NaDC1* mRNA levels were determined by RT-PCR. As shown in Figure 5, no modifications were observed in Oat5 mRNA and in *NaDC1* mRNA levels in kidney from BDL group as compared with Sham ones.

DISCUSSION

Cholestasis has been demonstrated to alter the transport of compounds such as bile salts and of other organic anions in liver and kidneys[11,12]. Renal function is also impaired during cholestasis as previously reported[10,13,15,18]. In this study we observed an increase in mRBF in BDL rats. This increase might lead to an important wash out of the cortico-medullar

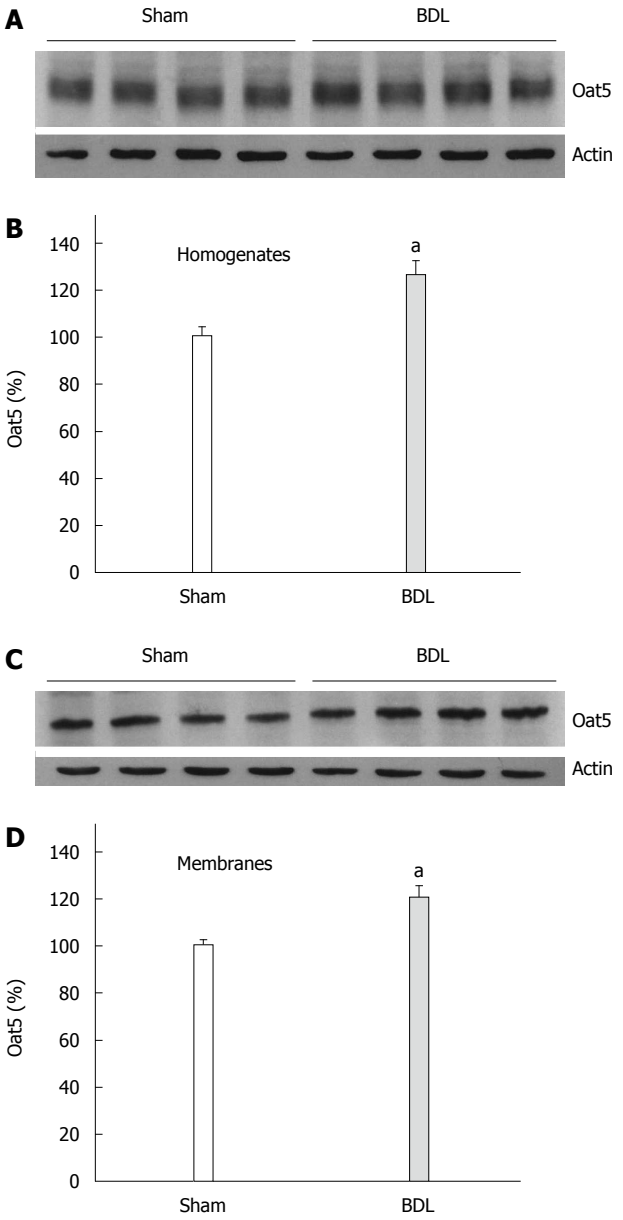

Figure 1 Oat5 protein expression in homogenates. Renal homogenates (20 μg proteins) (A) and brush border membranes (10 μg proteins) (C) from kidneys of Sham and BDL rats were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (8.5%) and blotted onto nitrocellulose membranes. Oat5 was identified using a non-commercial polyclonal antibody as described in Materials and Methods. Densitometric quantification of Oat5 (B and D). Sham levels were set at 100%. Each column represents mean ± SE from experiments carried out in triplicate on four different homogenates and brush border membranes preparations for each experimental group, $^{a}P < 0.05$ *vs* Sham.

gradient. Moreover, we found an increased urinary volume that might be explained by the wash out of the cortico-medullar gradient and by the presence in the urine of a greater amount of osmotically active solutes such as bile acids[24,25] that could not be eliminated by the liver. This finding suggests intrarenal blood flow distribution with increased medullary blood flow following acute bile duct ligation as other authors have described after chronic bile duct ligation in rats[10].

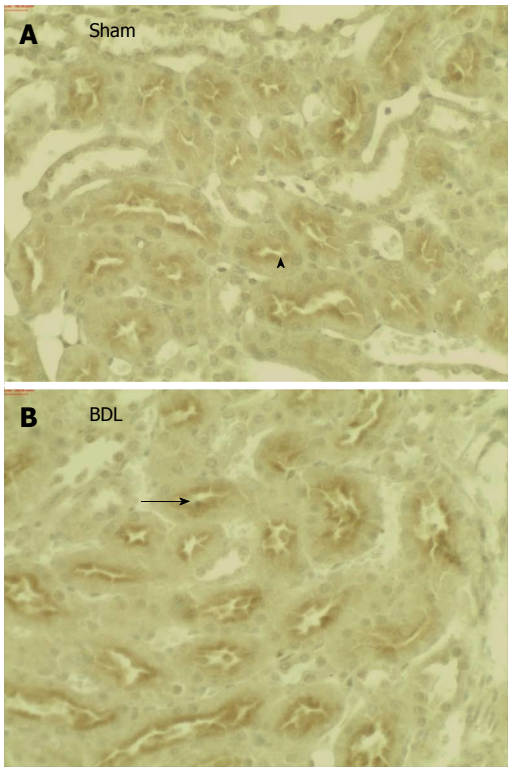

Figure 2 Immunohistochemistry for Oat5 in kidneys from Sham (A) and BDL (B) rats. Serial sections from each rat kidney were stained using a non-commercial anti-Oat5 antibody. Oat5 labeling was associated with the apical plasma membrane domains in proximal tubule cells (arrow heads). In kidneys from BDL group there was a marked increase in Oat5 staining (arrows). These figures are representative of typical samples from four rats for each experimental group. Magnification × 200.

In this study we observed no differences in the biochemical parameters evaluated in urine, glucose and protein urinary excretion and the activity of alkaline phosphatase. At this point it is important to emphasize that there was no difference in the glomerular filtration rate, evaluated in this study by creatinine clearance, between Sham and BDL rats as it was previously described by us using inulin clearance[15].

We have previously demonstrated alterations on the expression of different transporters in rats suffering extrahepatic cholestasis[12-15,18].

The organic anion/dicarboxylate exchanger Oat5 is important for the transport of several organic anions, including steroids sulphates[26]. Little is known about renal expression of Oat5 under pathological conditions, particularly under obstructive jaundice. In this experimental model of extrahepatic cholestasis, we found an increase in Oat5 protein expression both in BBM and in homogenates in BDL group. In addition to immunoblotting, the immunohistochemical technique corroborated the increase in apical membrane expression of Oat5. No modifications were observed in mRNA levels for *Oat5*. These results suggest a decrease in Oat5 protein degradation. The Oat5 upregulation might lead to a higher elimination/reabsorption of its transported compounds. This is

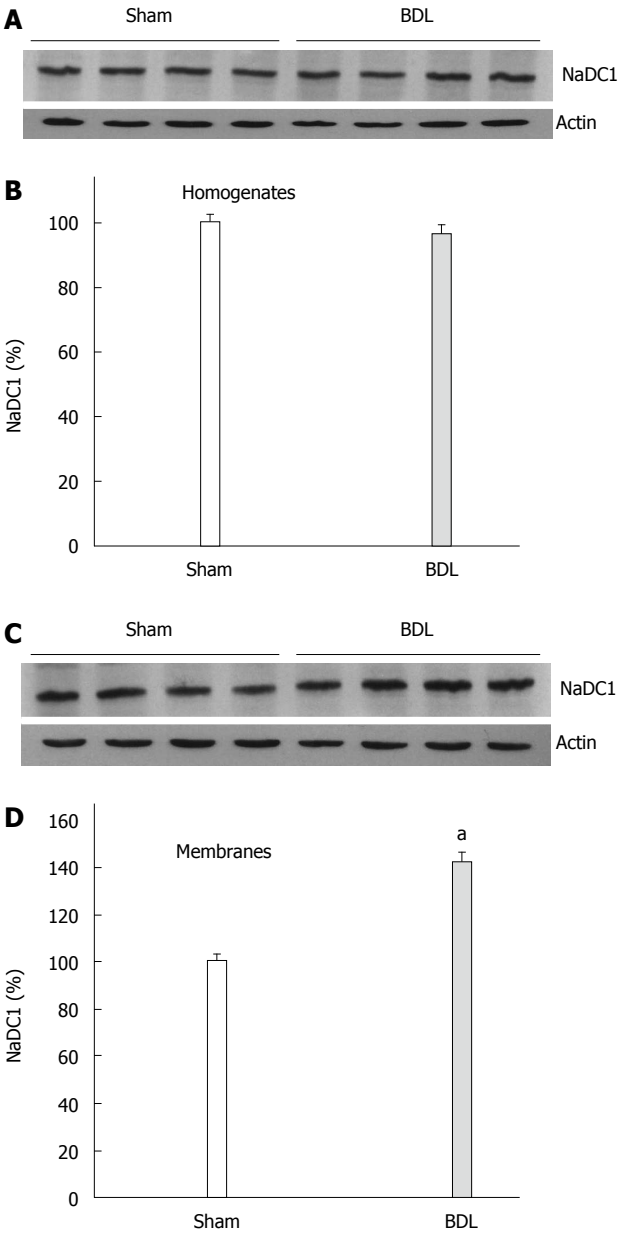

Figure 3 NaDC1 protein expression in homogenates. Renal homogenates (20 μg proteins) (A) and brush border membranes (10 μg proteins) (C) from kidneys of Sham and BDL rats were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (8.5%) and blotted onto nitrocellulose membranes. NaDC1 was identified using a non-commercial polyclonal antibody as described in Materials and Methods. Densitometric quantification of NaDC1 (B and D). Sham levels were set at 100%. Each column represents mean ± SE from experiments carried out in triplicate on four different homogenates and brush border membranes preparations for each experimental group, $^{a}P < 0.05$ *vs* Sham.

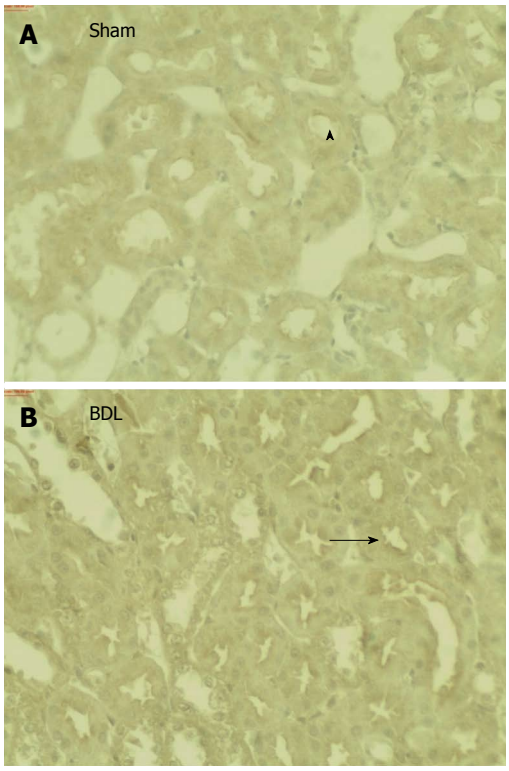

Figure 4 Immunohistochemistry for NaDC1 in kidneys from Sham (A) and BDL (B) rats. Serial sections from each rat kidney were stained using a non-commercial anti-NaDC1 antibody. NaDC1 labelling was associated with the apical plasma membrane domains in proximal tubule cells (arrow heads). In kidneys from BDL group there was a marked increase in NaDC1 staining (arrows). These figures are representative of typical samples from four rats for each experimental group, magnification × 200.

especially important for those drugs transported by this protein because their pharmacokinetics may be altered during cholestasis.

Sodium-coupled transporters, such as NaDC1, are responsible for the active transport of Krebs cycle intermediates, including succinate, α-ketoglutarate and citrate[7]. In this study, we observed that BDL rats have a higher renal expression of NaDC1 protein at apical membranes while no difference is observed in homogenates which might suggest an increased recruitment of preformed transporters into the membranes or an inhibition in the internalization of membrane transporters. Immunohistochemical technique corroborated these results, showing an increase in the staining for NaDC1 in apical membranes of proximal tubule cells from BDL animals. No modifications were observed in mRNA levels for *NaDC1*.

It has been proposed that NaDC1 and Oat5 have an important role in the late (S2 to S3) segments of proximal tubules. Using the outward gradient of succinate, citrate and α-ketoglutarate generated by NaDC1, Oat5 can reabsorb some organic anions, such as steroid sulphates that are glomerular filtrated or tubular secreted by multidrug resistance proteins such as Mrp2 and Mrp4[4]. NaDC1 cotransporter might also help to maintain the outward dicarboxylate gradient necessary for the correct function of Oat proteins both at apical (Oat5) and basolateral membranes (Oat1 and Oat3)[27]. Thus, Oat1 and Oat3 may contribute to the elimination of toxic metabolites such as bile acids and other potential toxins existing in extrahepatic cholestasis that could not be excreted into bile in this cholestatic model. Then, Oat5 may contribute to the elimination of these compounds as well as anionic drugs that are uptaken by Oat1 and Oat3.

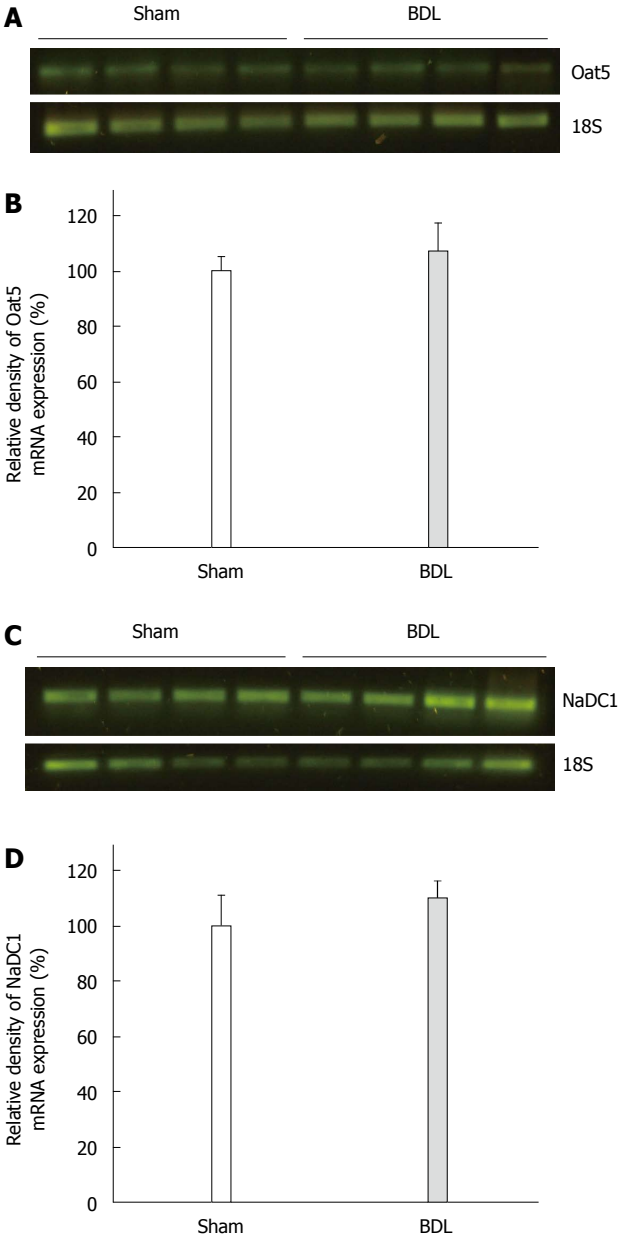

Figure 5 Expression of *Oat5* (A, B) and *NaDC1* (C, D) mRNA in the kidney from Sham and BDL rats. Data are presented as percentage considering the mean Sham value as 100%. Results are expressed as mean ± SE of four rats per group. The 18S mRNA was used as internal control.

Besides the upregulations of both apical proteins NaDC1 and Oat5, we have found an important decrease in citrate urine levels in BDL rats, at least in part, because of higher tubular citrate reabsorption.

The importance of extracellular or luminal pH in the alteration of citrate reabsorption is also emphasized[7,9]. We found an increase in urinary excretion of H^+ in BDL rats that would favour the protonation of $citrate^{3-}$ to $citrate^{2-}$ making it a much stronger substrate for the dicarboxylate transporters[7]. Furthermore, this increase in urinary excretion of H^+ in BDL rats might explain, at least in part, the increased renal expression of NaDC1 observed in BDL rats since it has been reported that this cotransporter is modulated by pH changes[7,9,28]. The accumulation of bilirubin, bile acids and other potential toxics existing in this experimental model of extrahepatic cholestasis may affect post-transcriptional mechanisms[29,30].

In summary, we present evidence that cholestasis induced by common bile duct ligation in the rat induces upregulations of both apical proteins Oat5 and NaDC1 in kidneys. The urinary excretion of citrate is decreased in BDL rats, probably because of higher NaDC1 expression. These modifications might be part of a likely adaptation leading to support normal renal tubular function by increasing important metabolites reabsorption, such as citrate, that have different important roles in proximal tubule cell metabolism. Besides, special attention might be paid for those drugs transported by Oat5 because their pharmacokinetics may be altered during cholestasis.

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COMMENTS

Background

Jaundice in chronic bile duct-ligated rats has been associated with functional and metabolic disturbances of the kidney. Altered absorption, distribution and elimination of drugs have been described in this pathology.

Research frontiers

Organic anion transporter 5 (Oat5) is a protein exclusively localized in the kidney. Oat5 has been reported to interact with many anionic drugs of pharmacological interest. The function of Oat5 in renal cells under pathological conditions has not been fully elucidated yet. The sodium-dicarboxylate cotransporter 1 (NaDC1) is also located in the kidney. The primary function of this transporter is to reabsorb filtered Krebs cycle intermediates. These compounds, such as succinate, citrate and α-ketoglutarate are important substrates for renal metabolism because they account for 10%-15% of oxidative metabolism in the kidney.

Innovations and breakthroughs

The effects of acute extrahepatic cholestasis on the expression of Oat5 and NaDC1 in rats, and the contribution of these effects on renal excretion of citrate were examined in this basic research study.

Applications

This study shows that cholestasis induces upregulations of both apical proteins Oat5 and NaDC1 in kidneys and decreases the urinary excretion of citrate. Thus, special attention might be paid for the renal handling of citrate and of those therapeutic drugs transported by Oat5 because their plasma levels may be altered during cholestasis.

Terminology

Cholestasis is a condition where bile cannot flow from the liver to the duodenum. The obstructive type of cholestasis is a mechanical blockage in

the duct system that can occur from a gallstone or malignancy. A membrane transport protein is a membrane protein involved in the movement of ions, small molecules or macromolecules across a biological membrane. Krebs cycle or citric acid cycle is a series of chemical reactions used by all aerobic organisms to generate energy through the oxidation of acetate derived from carbohydrates, fats and proteins into carbon dioxide and chemical energy in the form of adenosine triphosphate.

Peer-review

This is a good descriptive study with important findings. Oat5 and NaDC1 are two carriers expressed in the apical membrane of renal proximal tubule cells. Oat5 is an organic anion/dicarboxylate exchanger which has impact on renal excretion of hormones, drugs and xenobiotics. The primary function of NaDC1 is to reabsorb filtered Krebs cycle intermediates, such as citrate. In the present work, they found upregulations of both transporters and a decrease in urinary citrate excretion in bile duct-ligated rats. Citrate excretion is decreased at least in part, because of the higher NaDC1 expression. Using the outward gradient of citrate generated by NaDC1, Oat5 can reabsorb/eliminate different organic anions of pathophysiological importance. Attention might be paid for those drugs transported by this protein because their pharmacokinetics may be altered during cholestasis.

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ORIGINAL ARTICLE

Basic Study

Brewers' rice modulates oxidative stress in azoxymethane-mediated colon carcinogenesis in rats

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Author contributions: Tan BL, Norhaizan ME, Yeap SK and Roselina K designed the study; Tan BL carried out the experiments; Tan BL and Huynh K performed acquisition of data; Tan BL, Norhaizan ME and Hazilawati H performed the statistical analysis and interpretation of data; Tan BL and Norhaizan ME drafted the manuscript and revised the manuscript critically; all authors read and approved the final version of the manuscript.

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Institutional review board statement: This study was conducted following the guidelines approved by the Institutional Animal Care and Use Committee of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia Serdang, Selangor.

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia Serdang, Selangor (IACUC protocol number: UPM/FPSK/PADS/BR-UUH/00461).

Conflict-of-interest statement: The authors declare that they have no conflicts of interest.

Data sharing statement: No additional data are available.

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Abstract

AIM: To investigate the mechanistic action of brewers' rice in regulating the Wnt/nuclear factor-kappa B (NF-κB)/Nrf2-signaling pathways during colon carcinogenesis in male Sprague-Dawley rats.

METHODS: Male Sprague-Dawley rats were randomly divided into the following five groups (six rats in each group): (G1) normal, (G2) azoxymethane (AOM) alone, (G3) AOM + 10% (weight (w)/weight (w)) brewers' rice, (G4) AOM + 20% (w/w) brewers' rice, and (G5) AOM + 40% (w/w) brewers' rice. They were intraperitoneally administered 15 mg/kg body weight of AOM in saline once weekly over a two-week period and treated with an American Institute of Nutrition (AIN)-93G diet containing 10%, 20%, and 40% (w/w) brewers' rice. The mRNA levels of glycogen synthase kinase 3β (*GSK*3β), β-catenin, key inflammation markers, nuclear factor E2-related factor 2 (*Nrf2*), and heme oxygenase-1 (*HO-1*)-dependent transcriptional activity were assessed by quantitative real-time polymerase chain reaction analyses. The colon superoxide dismutase, malondialdehyde, and nitric oxide levels were also analyzed to assess the antioxidant effect of these treatments. The results were analyzed using one-way analysis of variance (ANOVA), and a *P* value of < 0.05 was considered significant.

RESULTS: The overall analyses demonstrated that the dietary administration of brewers' rice in AOM-induced rat colon carcinogenesis resulted in the transcriptional upregulation of *GSK*3β, inducible nitric oxide synthase (*iNOS*), *Nrf2*, and *HO-1*. We discovered that the dietary administration of brewers' rice downregulated the β-catenin and NF-κB mRNA levels. A significant reduction in β-catenin expression was found in the groups administered with 20% (0.611 ± 0.034) and 40% (0.436 ± 0.045) (w/w) brewers' rice compared with that of the group treated with AOM alone (1.000 ± 0.064) (*P* < 0.05). The NF-κB expression was significantly lower between the AOM-alone group (1.000 ± 0.048) and those groups fed with diets containing 10% (w/w) brewers' rice (0.255 ± 0.022), 20% (w/w) brewers' rice (0.450 ± 0.045), or 40% (w/w) brewers' rice (0.541 ± 0.027) (*P* < 0.05). Brewers' rice improved the antioxidant levels, indicating that brewers' rice can enhance effective recovery from oxidative stress induced by AOM.

CONCLUSION: Our results provide evidence that brewers' rice can suppress colon cancer *via* the regulation of Nrf2 expression and the inhibition of the Wnt/NF-κB signaling pathways.

Key words: Brewers' rice; Nuclear factor-kappa B; Colon cancer; β-catenin; Nuclear factor E2-related factor 2

Core tip: This study demonstrates that a treatment with 40% (w/w) brewers' rice modulated the Wnt signaling pathway. Feeding 20% (w/w) brewers' rice markedly improved the antioxidant level. These results strongly imply the potential use of brewers' rice in future applications to combat oxidative stress and colon cancer.

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INTRODUCTION

Colorectal cancer has become the third most prevalent cancer after lung and breast cancers and contributes to nearly 10% of the total cases of cancer and approximately 8% of total cancer deaths worldwide[1]. It represents the third and second most commonly diagnosed cancer in males and females, respectively, with more than 1.2 million new cancer cases and 608700 deaths in 2008 worldwide[2].

The deregulation of Wnt/β-catenin signaling has been demonstrated to be associated with cancer, particularly colorectal cancer[3]. Chronic infection and inflammation promote the expression of nuclear factor-kappa B (NF-κB)[4] and inflammatory-associated genes, such as inducible nitric oxide synthase (*iNOS*)[5]. The NF-κB pathway is associated with colorectal cancer, and the inhibition of NF-κB activation can reduce chemoresistance[6]. The nuclear factor E2-related factor 2 (Nrf2) transcription factor signaling pathway has become a target for chemoprevention. A previous study reported that Nrf2 regulates the expression of numerous detoxifying and antioxidant enzymes toward oxidative or electrophilic stress[7].

The health benefits of natural products have led to their recognition as sources of remedy[8]. Most studies have indicated that cancers may be prevented or delayed by treatment with natural dietary products or synthetic compounds[9]. Rice (*Oryza sativa* L.), an essential cereal crop grown in Asia, has become a major source of carbohydrates in the daily diet. Epidemiological studies have demonstrated that whole grain foods are recognized to be important for providing protection against cancer[10]. Brewers' rice, known locally as *temukut*, consists of broken rice, rice bran, and rice germ, which is a waste product of the rice industry. The production of brewers' rice during rice milling has been described in a previous report[11].

Our earlier study showed that the dietary administration of brewers' rice can reduce the risk of azoxymethane (AOM)-induced colon carcinogenesis in rats through the downregulation of β-catenin and cyclooxygenase (COX-2)[12]. However, the molecular mechanism underlying these effects remains obscure. We hypothesized that brewers' rice may provide chemopreventive or chemotherapeutic effects against colorectal cancer *via* regulation of multiple signaling pathways. The present study sets out to determine whether brewers' rice confers suppressive effects on the gene expression of β-catenin and key inflammation markers, such as NF-κB and *iNOS*, which are par-

ticularly critical in the development of colon cancer. Glycogen synthase kinase 3β (*GSK*3β), a destruction complex that modulates the degradation of β-catenin, was also evaluated. Moreover, the potential roles of brewers' rice in the regulation of *Nrf2*-dependent transcriptional activity were assessed during AOM-induced colon tumorigenesis in male Sprague-Dawley rats. *Nrf2* and heme oxygenase-1 (*HO-1*) were evaluated to determine the effect of brewers' rice in carcinogen metabolism against detoxification. The colon superoxide dismutase (SOD), malondialdehyde (MDA), and nitric oxide (NO) levels were also analyzed to assess the antioxidant effect of these treatments.

MATERIALS AND METHODS

Chemicals and reagents

AOM and phosphate-buffered saline (PBS) were purchased from Sigma-Aldrich (St. Louis, MO, United States). RNA Shield™ reagent was obtained from Zymo Research Corp. (Irvine, CA, United States). HiYield™ Total Ribonucleic Acid (RNA) Mini Kit (Tissue) was purchased from Real Biotech Corporation (Banqiao City, Taipei County, Taiwan). High Capacity RNA-to-cDNA Kit and SYBR® Select Master Mix (CFX) were purchased from Applied Biosystems (Foster City, CA, United States). Specific primers were purchased from Sigma-Aldrich (St. Louis, MO, United States). Griess Reagent Kit was obtained from Invitrogen™ (Carlsbad, CA, United States). All other chemicals and reagents used were of analytical grade and bought from Sigma-Aldrich (St. Louis, MO, United States).

Brewers' rice

Freshly milled brewers' rice samples from rice variety MR 219 were obtained from the BERNAS Milling Plant at Seri Tiram Jaya, Selangor, Malaysia. The stabilization of brewers' rice was conducted as previously reported by Tan *et al*[13].

Diet and animals

This study was conducted following the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM) Serdang, Selangor (IACUC protocol number: UPM/FPSK/PADS/BR-UUH/00461). A total of 30 four-week-old male Sprague-Dawley rats (*Rattus norwegicus*) were housed in a well-ventilated room at 25 to 27 ℃ with 50% ± 10% relative humidity and 12-h light/dark cycles. Hygienic conditions were maintained by weekly changes of woodchip beds. The animals were acclimatized for seven days and administered an American Institute of Nutrition (AIN)-93G diet and water *ad libitum*. The animals were randomly divided into the following five groups (six rats in each group): (G1) normal, (G2) AOM alone, (G3) AOM + 10% (weight (w)/weight (w)) brewers' rice, (G4) AOM + 20% (w/w) brewers' rice, and (G5) AOM + 40% (w/w) brewers' rice. Beginning at six weeks of age, the rats were intraperitoneally given injections of AOM at a dose of 15 mg/kg body weight once weekly over a two-week period, whereas the rats in the normal group were given normal saline (vehicle control). The control groups (G1 and G2) were fed an AIN-93G diet, and the G3, G4, and G5 groups were given an AIN-93G diet containing 10%, 20%, and 40% (w/w) brewers' rice, respectively. The experimental diets were prepared weekly and kept at 4 ℃. The composition of the experimental diet (Table 1) was adjusted according to the nutrient content of brewers' rice with respect to moisture (11.36% ± 0.12%), ash (1.56% ± 0.26%), protein (9.01% ± 0.27%), fat (1.95% ± 0.11%), total available carbohydrates (72.42% ± 1.25%), and total dietary fiber (5.32% ± 0.04%) contents[12]. After twenty weeks of treatment, the animals were sacrificed after anesthesia with diethyl ether, and the colon tissue was removed, rinsed with PBS, opened longitudinally, and fixed with RNA Shield™ reagent or stored at -20 ℃ for further analyses.

Total RNA extraction and cDNA synthesis

The extraction of total RNA from colon tissue was performed using the HiYield Total RNA Mini Kit (Tissue). Initially, colon tissue disruption and homogenization were performed according to the manufacturer's protocols. The colon tissue was homogenized in a mixture of 100 μL of lysis buffer, 400 μL of RB buffer, and 4 μL of β-mercaptoethanol. The sample was then incubated for 5 min at room temperature and centrifuged at 15680 × *g* for 5 min. The supernatant was passed through the filter column and the collection tube. After centrifugation at 93 × *g* for 1 min, 400 μL of 70% ethanol was added and passed through the RB column. After adding 400 μL of W1 Buffer and 600 μL of wash buffer, the RNA was eluted with 50 μL of RNase-free water and kept at -80 ℃. Two microliters of nuclease-free water was added to the pedestal for a blank sample. After that, 1 μL of RNA sample was added. The RNA concentration was measured at 260 nm using a nanophotometer. Two micrograms of total RNA per 20 μL was reverse-transcribed using the High Capacity RNA-to-cDNA Kit, according to the manufacturer's protocols. The reverse transcription reaction was performed using an Authorized Thermal Cycler. The reaction was performed at 37 ℃ for 60 min followed by 95 ℃ for 5 min to denature the enzyme and then maintained at 4 ℃. The cDNA was then ready for use as a template for the amplification of real-time polymerase chain reaction (PCR).

Quantitative real-time polymerase chain reaction analysis

The nucleotide primer sequences of rat origin were obtained from the National Center for Biotechnology Information Gene Bank (Table 2). The specific primers

Table 1 Composition of experimental diets

Ingredients (g/1000 g diet)	Group				
	G1	G2	G3	G4	G5
Brewers' rice	-	-	100.0	200.0	400.0
Corn starch	397.5	397.5	315.3	233.2	68.9
Casein	200.0	200.0	191.0	182.0	164.0
Maltodextrin	132.0	132.0	132.0	132.0	132.0
Sucrose	100.0	100.0	100.0	100.0	100.0
Soybean oil	70.0	70.0	68.1	66.1	62.2
Powdered cellulose	50.0	50.0	44.7	39.4	28.7
AIN-93G mineral mix	35.0	35.0	33.4	31.9	28.8
AIN-93G vitamin mix	10.0	10.0	10.0	10.0	10.0
L-cystine	3.0	3.0	3.0	3.0	3.0
Choline bitartrate	2.5	2.5	2.5	2.5	2.5
tert-butylhydroquinone	0.014	0.014	0.014	0.014	0.014

G1 and G2: AIN-93G diet; G3: AIN-93G diet containing 10% (w/w) of brewers' rice; G4: AIN-93G diet containing 20% (w/w) of brewers' rice; G5: AIN-93G diet containing 40% (w/w) of brewers' rice.

Table 2 Nucleotide sequence of polymerase chain reaction primers (obtained from GenBank database)

Primer name [Accession number]	Oligonucleotides (5'-3') Sequence
*GSK*3β	F: GGGCACCAGAGCTGATCTTT
[NM_032080]	R: GCCGAAAGACCTTCGTCCA
Beta-catenin	F: CGTGGAAGCTGGTGGGATG
[AF121265]	R: TTCCTGCTTAGTCGCTGCAT
NF-κB	F: AGAGGATGTGGGGTTTCAGG
[NM_001276711.1]	R: GCTGAGCATGAAGGTGGATG
iNOS	F: GTACCCTCAGTTCTGTGCCT
[NM_012611.3]	R: TGTTGCGTTGGAAGTGTAGC
*Nrf*2	F: TCTGACTCCGGCATTTCACT
[NM_031789.2]	R: CCCCAGAAGAATGTGTTGGC
HO-1	F: CTAGAGCAGGACATGGCCTT
[NM_012580.2]	R: GCCTTCTGCGCAATCTTCTT
ACTB[1]	F: CCACCCGCGAGTACAACC
[NM_031144.3]	R: TCAGGATGCCTCTCTTGCTC
B2M[1]	F: CCCACCCTCATGGCTACTTC
[NM_012512.2]	R: GATGAAAACCGCACACAGGC
*RPLP*1[1]	F: CAAGGTGCTCGGTCCTTCC
[NM_001007604.2]	R: GAGCCTTTGCAAACAAGCCA

[1]Housekeeping gene. *ACTB*: β-actin; *B2M*: Beta-2 microglobulin; *GSK*3β: Glycogen synthase kinase 3β; *HO-1*: Heme oxygenase-1; *iNOS*: Nitric oxide synthase, inducible; NF-κB: Nuclear factor-kappa B; *Nrf2*: Nuclear factor E2-related factor 2; *RPLP*1: Ribosomal protein, large, P1.

were validated for amplification specificity, amplification efficiency over a concentration range and consistency with the amplification efficiency of housekeeping genes. The mRNA levels of *GSK*3β, β-catenin, NF-κB, *iNOS*, *Nrf2*, and *HO-1* were assayed using SYBR® Select Master Mix, CFX in a final volume of 20 μL, according to the manufacturer's protocols. Initially, the cDNA template, primers, and kit contents (SYBR® Select Master Mix (CFX) and RNase-free water) were thawed on ice. Upon thawing, the reaction mix was prepared and thoroughly mixed. The qPCR reaction was then analyzed based on the following conditions: (1) uracil-DNA glycosylase (UDG) activation at 50 ℃ for 120 s (1 cycle); and (2) DNA polymerase activation at 95 ℃ for 120 s (1 cycle); denaturation at 95 ℃ for 2 s (40 cycles); and annealing/extension at 60 ℃ for 30 s (40 cycles). All samples and controls were determined in triplicate using an Eco™ Real-Time PCR system, and the v4.0.7.0 software (Illumina, Inc., San Diego, CA, United States) was used for data analysis. The fold inductions of the samples were compared with the control (AOM-alone group). Beta-actin (*ACTB*), β-2 microglobulin (*B2M*), and ribosomal protein, large, P1 (*RPLP1*) were used as housekeeping genes to normalize the expressions of the target genes.

Colon tissue preparation

The colon tissues of rats were homogenized in ice-cold PBS. Supernatants were collected by centrifugation at 370 × *g* and 4 ℃ for 5 min and stored at -80 ℃ for the SOD[14], MDA[15], and NO[16] assays.

Determination of superoxide dismutase

The SOD levels in the colon homogenates were analyzed following the inhibition of the reduction of nitroblue tetrazolium (NBT). Tissue supernatant was mixed with 0.1 mol/L of ethylenediaminetetraacetic acid (EDTA), 0.15 mg/mL of sodium cyanide, 1.5 mmol/L of NBT, 0.12 mmol/L of riboflavin, and 0.067 mol/L of phosphate buffer in a 300 μL volume. The sample absorbance was read at 560 nm, and the percentage of SOD inhibition was compared with that of the blank. The concentration of the sample was calculated using the amount of protein required to achieve 50% inhibition and expressed as U/mg of protein.

Determination of malondialdehyde

Lipid peroxidation was determined by measuring the thiobarbituric acid reactive substance (TBARS) levels. An aliquot of 100 μL of the supernatant was diluted with 400 μL of PBS and added with 12.5 μL of butylated hydroxytoluene (BHT, 8.8 mg/mL) and 250 μL of trichloroacetic acid (TCA, 30%). The mixture was vortexed, allowed to stand for 2 h at 4 ℃ and centrifuged at 2000 × *g* for 15 min. The supernatant

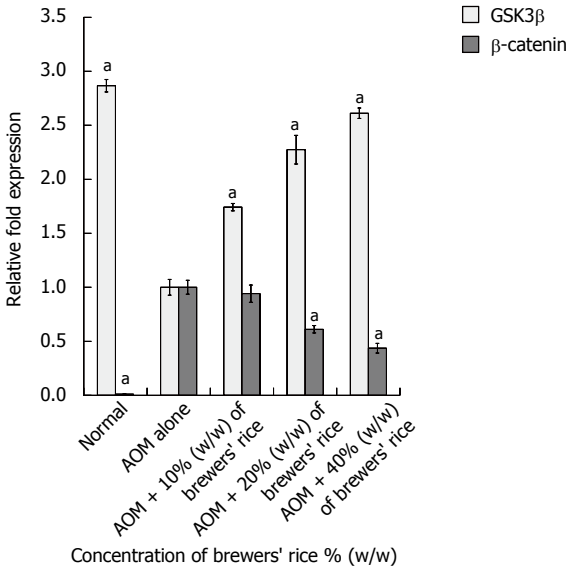

Figure 1 mRNA levels of *GSK3*β and β-catenin in AOM-injected colon cancer treated with brewers' rice (*n* = 3). $^{a}P < 0.05$ *vs* AOM alone, Tukey's test. AOM: Azoxymethane; *GSK*3β: glycogen synthase kinase 3β.

was boiled for 15 min with 37.5 μL of 0.1 mol/L EDTA and 125 μL of thiobarbituric acid (TBA, 1%). After cooling at room temperature, the absorbance of the pink-colored product was measured at 532 and 600 nm using an ELISA Reader (BioTek Instruments, Inc., Tigan Street, Winooski, United States). An aqueous solution of tetramethoxypropane was used as the standard. The MDA level was expressed as nmol MDA/g of protein and determined using a standard curve.

Determination of nitric oxide

NO production in the colon was evaluated using a colorimetric Griess Reagent Kit, according to the manufacturer's protocols. A 100-μL aliquot of the colon supernatant was loaded in the microtiter plate, and 20 μL of Griess reagent [0.1% of N-(1-naphthyl)ethylenediamine dihydrochloride and 1% of sulfanilic acid in 5% phosphoric acid] and 80 μL of deionized water were then added. The absorbance was measured at 540 nm using an ELISA Reader (BioTek Instruments, Inc., Tigan Street, Winooski, United States).

Statistical analysis

Data are expressed as mean ± SD, and statistical analyses were performed using one-way analysis of variance (ANOVA). Differences with $P < 0.05$ were considered significant. The statistical analyses were performed using the Statistical Package for Social Science (SPSS) version 19.0.

RESULTS

Brewers' rice promotes GSK3β mRNA level in colon tumorigenesis

In the current study, we determined the *GSK*3β mRNA level of the control (normal and AOM alone) groups and the treatment groups through quantitative real-time PCR analyses. We observed that the normal group (2.866 ± 0.058) had the highest *GSK*3β mRNA level compared with the brewers' rice-fed groups (Figure 1). The administration of brewers' rice significantly increased the transcription of the *GSK*3β gene compared with AOM alone ($P < 0.05$). These findings clearly demonstrated that the dietary administration of brewers' rice in AOM-induced rat colon carcinogenesis resulted in a dose-dependent increase in the *GSK*3β mRNA level.

Brewers' rice inhibits the β-catenin pathway in colonic tumors

As shown in Figure 1, our results showed that the colonic tumors in the groups treated with AOM alone had the highest β-catenin mRNA levels, whereas the administration of 20% (0.611 ± 0.034) and 40% (0.436 ± 0.045) (w/w) brewers' rice markedly decreased the β-catenin mRNA levels. A significant reduction in β-catenin expression was found in the groups administered with 20% and 40% (w/w) brewers' rice compared with the group treated with AOM alone ($P < 0.05$). In brewers' rice-treated AOM-injected colon tumorigenesis rats, the phosphorylation and degradation of β-catenin increased in a dose-dependent manner. A very low β-catenin amount was observed in the normal group (0.011 ± 0.003) (Figure 1).

Brewers' rice inhibits the expression of NF-κB in colon tumorigenesis

We hypothesized that brewers' rice downregulates the expression of NF-κB. As expected, none of the rats exhibited NF-κB expression in the normal colon mucosa (Figure 2). The overall analysis indicated that the colon tissue in the group treated with AOM alone presented the highest NF-κB expression (1.000 ± 0.048) compared with the groups treated with brewers' rice. A significant reduction in the gene expression of NF-κB was also observed in the rats of the groups treated with brewers' rice compared with the group treated with AOM alone ($P < 0.05$). This finding revealed that the administration of brewers' rice resulted in the inhibition of NF-κB expression, and the maximum effect was obtained with 10% (w/w) brewers' rice (0.255 ± 0.022).

Brewers' rice upregulates the iNOS mRNA level in colon tumorigenesis

In the present study, we observed a high expression of *iNOS* mRNA in the normal colon mucosa (9.134 ± 0.708). The data presented in this study demonstrated that the groups administered with 20% (9.090 ± 0.519) and 40% (8.582 ± 1.261) (w/w) brewers' rice exhibited significantly upregulated *iNOS* mRNA levels compared with the group treated with AOM alone ($P < 0.05$) (Figure 2).

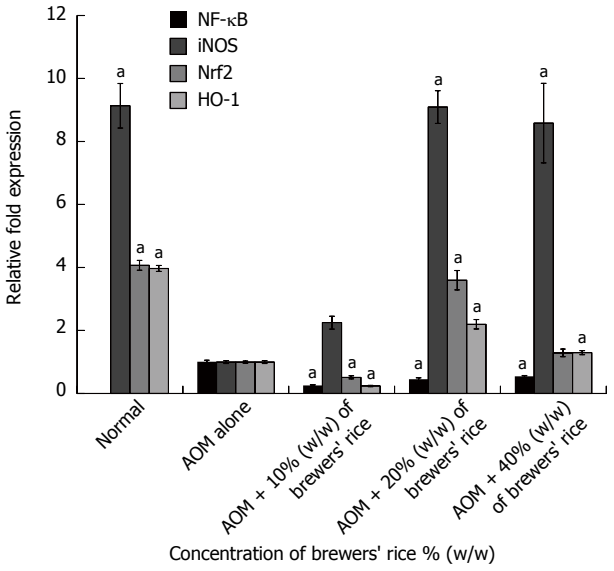

Figure 2 mRNA levels of nuclear factor-kappa B, inducible nitric oxide synthase, nuclear factor E2-related factor 2, and heme oxygenase-1 in azoxymethane-injected colon cancer treated with brewers' rice (*n* = 3). [a]*P* < 0.05 *vs* AOM alone, Tukey's test. AOM: Azoxymethane; *HO-1*: Heme oxygenase-1; *iNOS*: Inducible nitric oxide synthase; NF-κB: Nuclear factor-kappa B; *Nrf2*: Nuclear factor E2-related factor 2.

Brewers' rice activates the Nrf2 mRNA level in colon tumorigenesis

As shown in Figure 2, in the normal group, which was administered saline but not treated with brewers' rice, prominent *Nrf2* gene expression was observed in the normal colon mucosa (4.068 ± 0.155). Our results showed that treatment with 20% (w/w) brewers' rice (3.596 ± 0.308) effectively activated the gene expression of *Nrf2* compared with AOM alone (Figure 2).

Brewers' rice induces Nrf2-regulated HO-1 expression in colonic tumors

In the present study, we showed that the group treated with 20% (2.196 ± 0.150) and 40% (1.295 ± 0.063) (w/w) brewers' rice exhibited upregulated *HO-1* expression in colonic tumor tissue. Expectedly, we found that the normal group (3.967 ± 0.094) presented the highest expression of *HO-1* (Figure 2).

Effect of brewers' rice on the SOD, MDA, and NO levels in colon homogenate

The changes in the colon SOD, MDA, and NO activities after the dietary administration of brewers' rice on AOM-induced colon carcinogenesis are summarized in Table 3. The SOD levels in the two treatment groups [20% (61.71 ± 2.36 U/mg of protein) and 40% (61.29 ± 4.32 U/mg of protein) (w/w) of brewers' rice] were significantly elevated compared with that of the group treated with AOM alone (43.43 ± 2.96 U/mg of protein) ($P < 0.05$) (Table 3).

In addition to the effects on the SOD level, our findings showed that the highest MDA level was obtained in the group treated with AOM alone (18.01 ± 1.43 nmol/g of protein) compared with the groups treated with brewers' rice. A significant reduction in the MDA level was found in the two treatment groups (20% (14.24 ± 0.58 nmol/g of protein) and 40% (8.14 ± 1.42 nmol/g of protein) (w/w) of brewers' rice) compared with that of the group treated with AOM alone (18.01 ± 1.43 nmol/g of protein) ($P < 0.05$). These findings indicated that the dietary administration of brewers' rice resulted in reductions in the MDA level in a dose-dependent manner, and the maximum effect was obtained with a concentration of 40% (w/w) brewers' rice (8.14 ± 1.42 nmol/g of protein) (Table 3).

Consistent with the high levels of MDA observed in colon tumors, we also observed the highest NO level in the group treated with AOM alone (798.46 ± 30.45 μmol/mg of protein) compared with those of the other treatment groups (Table 3). After twenty weeks of treatment with brewers' rice, the NO level was reduced. The suppressive effect of brewers' rice on NO was notable in rats that received 20% (w/w) brewers' rice (533.40 ± 40.43 μmol/mg of protein).

DISCUSSION

The current study is an extension of our earlier work, which determined that brewers' rice was an effective dietary agent for the reduction of tumor incidence and multiplicity in rat colons induced with AOM[12]. We also determined that brewers' rice markedly suppressed β-catenin expression in both the cytoplasm and the nucleus[12]. In the present study, male Sprague-Dawley rats were given different doses [10%, 20%, and 40% (w/w)] of brewers' rice. A dosage of 10% (w/w) brewers' rice was administered as suggested by a previous study performed by Boateng *et al*[17] on rice bran and rice germ. This dosage has been reported to reduce tumor formation. Moreover, higher concentrations [20% and 40% (w/w) brewers' rice] were also used to determine the dose-dependent effect of brewers' rice as a dietary agent in a rat colon cancer experimental model. Our earlier study reported that the highest dose [40% (w/w) brewers' rice] was well-tolerated and did not suppress the growth of rats[12].

Targeting Wnt signaling upstream of T-cell factor (TCF)/β-catenin signaling is a critical therapeutic option. In the β-catenin destruction complex, *GSK*3β is one of the crucial components that modulates the degradation or accumulation of β-catenin in the nucleus. To ascertain whether brewers' rice modulated *GSK*3β *via* Wnt/β-catenin signaling, the *GSK*3β mRNA level was analyzed in the colon of rats induced with AOM. Overall, treatment with brewers' rice resulted in an increase in the *GSK*3β mRNA level, and the maximum effect was obtained with 40% (w/w) brewers' rice. To further verify whether the mechanisms of action of *GSK*3β observed in the colons of rats injected with AOM suppressed

Table 3 Colon superoxide dismutase, malondialdehyde, and nitric oxide levels in azoxymethane-induced colon cancer after twenty weeks treatment

Group	SOD (U/mg of protein)	MDA (nmol/g of protein)	NO (μmol/mg of protein)
Normal	60.93 ± 5.23[a]	5.36 ± 0.23[a]	414.64 ± 11.59[a]
AOM alone	43.43 ± 2.96	18.01 ± 1.43	798.46 ± 30.45
AOM + 10% (w/w) of brewers' rice	43.85 ± 6.32	17.34 ± 3.16	622.70 ± 15.62[a]
AOM + 20% (w/w) of brewers' rice	61.71 ± 2.36[a]	14.24 ± 0.58[a]	533.40 ± 40.43[a]
AOM + 40% (w/w) of brewers' rice	61.29 ± 4.32[a]	8.14 ± 1.42[a]	619.35 ± 15.04[a]

Each value expressed as mean ± SD (n = 3). [a]P < 0.05 *vs* AOM alone, Tukey's test. MDA: Malondialdehyde; NO: Nitric oxide; SOD: Superoxide dismutase.

β-catenin expression, the mRNA level of β-catenin in response to brewers' rice was further analyzed.

The Wnt/β-catenin pathway plays a vital role in tissue homeostasis and cancer susceptibility. The dysregulation of β-catenin and other Wnt molecules results in the nuclear localization of β-catenin, stimulation of Wnt target genes, and tumor formation[18]. Mutations in the β-catenin gene are usually found in AOM-induced colon tumorigenesis in rats and mice[19]. These findings, which are supported by the current data, further indicate that the activation of the β-catenin gene plays a vital role in the development of colon tumors in rats. The finding that the depletion of β-catenin suppresses tumor incidence and multiplicity in brewers' rice-treated AOM-induced colon tumorigenesis suggests that brewers' rice may become a potential strategy for the therapeutic control of Wnt/β-catenin signaling in colon cancer. In the present study, treatments with brewers' rice resulted in increased *GSK*3β and decreased β-catenin, and the maximum effect was observed with 40% (w/w) brewers' rice. The effects observed in the treatment with 40% (w/w) brewers' rice could be explained by its higher concentrations of active compounds in brewers' rice, which may confer better functional properties in the regulation of Wnt/β-catenin signaling pathway. A very low β-catenin mRNA level observed in the normal group was consistent with the findings reported by Barker *et al*[20], who found that Wnt/β-catenin signaling played an essential role in intestinal development, which is specific for the intestinal and mammary epithelia. A previous study also demonstrated that most of the β-catenin protein was present at very low amounts in the cytoplasm or nucleus of normal cells[21]. Cytoplasmic β-catenin was maintained at a low level for tissue homeostasis, particularly in strongly proliferative, self-renewing tissues, such as the skin and gut[22]. However, Wnt pathway mutations are not the only factors that promote the activation of β-catenin[23]. A study reported that NF-κB also plays a crucial role in colorectal and colitis-associated tumorigenesis[24]. Aberrant NF-κB stimulation has been identified in more than 50% of colorectal and colitis-associated tumors[25]. Thus, the expression levels of NF-κB in response to brewers' rice were evaluated in the colons of rats induced with AOM.

The NF-κB family is a group of inducible transcription factors that are involved in immune and inflammatory responses and inhibit cell apoptosis. A previous study revealed that cancer cells with activated NF-κB are resistant against chemotherapeutics and ionizing radiation and that suppression of NF-κB activity markedly increases the sensitivity of cells to chemotherapeutic agents[26]. The inhibition of NF-κB transcriptional activity resulting from the administration of brewers' rice was further supported by Biswas *et al*[27] and Xie *et al*[28], who found that phenolic compounds inhibited NF-κB in cell cultures and promoted anti-inflammatory and antioxidant responses. Although the maximum effect was observed in 10% (w/w) brewers' rice, there was no significant difference between groups fed with 10% (w/w) brewers' rice and groups fed with diets containing 20% (w/w) brewers' rice or 40% (w/w) brewers' rice (P > 0.05). The reason for the lack of any clear dose-dependence effects remains to be elucidated. One of the possible reasons may be due to the efficiency of brewers' rice involved in the inhibition of NF-κB transcriptional activity reached with 10% (w/w) brewers' rice. Collectively, the data presented in this study suggest that brewers' rice may modulate colon tumor development through NF-κB signaling. In addition to the effects observed in Wnt and NF-κB signaling, the role of *iNOS* in the suppression of colon tumorigenesis elicited by brewers' rice remains unknown. Therefore, we further determined the chemoprevention mechanism of *iNOS* on brewers' rice in this model.

NO is produced during transcription and translation *via* iNOS, and once active, iNOS synthesizes high NO levels until substrate depletion[29]. However, our study shows contradictory results. It is possible that multiple cellular factors affect the sensitivity of NO, like specific NO metabolism pathways and interactions with other free radicals. The sensitivity of NO may also be associated with the expression of apoptosis-associated proteins, including Bcl-2, Bax, and Fas[30]. Excessive NO production can decrease the concentration of DNA repair enzymes and inhibit apoptosis through the nitrosylation of caspases[31]. The upregulation of *iNOS* mRNA levels in the current study was consistent with the results obtained by Radomski *et al*[32] and Dong *et al*[33], who reported that the expression of iNOS was

inversely associated with metastatic activity in human colon cancer and murine melanoma (K-1735) cells. This finding was further supported by Shi *et al*[34], who demonstrated that iNOS overexpression not only attenuated the proliferation and metastasis of human renal cell carcinomas and murine fibrosarcoma but also induced apoptosis. However, the study conducted by Shi *et al*[34] contradicted the results reported by Sheng *et al*[35] and Di Popolo *et al*[36], who demonstrated that elevated iNOS mRNA and protein levels partially contributed to the inhibition of apoptosis in colon cancer cells. Therefore, the activation of *iNOS* at the mRNA level may play a critical role in growth inhibition and apoptosis in a human colorectal cancer (HT-29) cell line, as determined in our earlier studies[13,37]. A previous study showed that Nrf2 enhanced the basal expression of cytoprotective genes and suppressed cytokine-mediated inflammation[38]. Thus, the expression of *Nrf2* in AOM-induced colon tissue was evaluated to determine whether brewers' rice could modulate *Nrf2* at the mRNA level.

Nrf2, which belongs to the Cap'n'Collar family of basic region-leucine zipper transcription factors, was shown to be a key element in the antioxidant response element (ARE)-mediated transcriptional machinery[39]. Nrf2 plays a crucial role in the regulation of phase Ⅱ detoxifying and antioxidant enzymes *via* AREs[40]. To determine whether brewers' rice decreased colorectal cancer by modulating the antioxidant-mediated pathway, we examined the transcription of *Nrf2*. Treatment with 20% and 40% (w/w) brewers' rice effectively activated the gene expression of *Nrf2* and may be associated with the modulation of xenobiotic-metabolizing enzymes and responsible for the balance of carcinogen metabolism against detoxification[41].

Nrf2 is stimulated by an oxidative signal in the cytoplasm, which allows its translocation to the nucleus where it interacts with DNA ARE regions and promotes the expression of cytoprotective enzymes, such as glutathione S-transferase (GST), SOD, HO-1, and NADPH-quinone oxidase (NQO) (ARE-regulated genes)[42]. Our findings indicated that the manipulation of brewers' rice in colonic tumor leads to changes in the gene expression of *Nrf2*-regulated *HO-1*, further suggesting that brewers' rice is a positive regulator of Nrf2 signaling. The transcriptional downregulation of β-catenin and *NF-κB* in carcinogen-injected rats after treatment with a brewers' rice diet was hypothesized because the carcinogen metabolism may have been shifted *via* Nrf2 and HO-1 in the colon. Our present study suggests that the possible chemopreventive mechanisms of brewers' rice against colon carcinogenesis may be associated with both the phase Ⅰ and Ⅱ drug-metabolizing enzymes regulated by Nrf2, thus resulting in the detoxification of AOM and the rapid metabolism of AOM by P450. Collectively, this finding suggests that brewers' rice may represent a promising natural dietary agent for the transcriptional downregulation of β-catenin and NF-κB and the upregulation of *Nrf2* and *HO-1* levels.

In addition to the effects observed in *Nrf2* and *HO-1* activation, the upregulation of *Nrf2* and *HO-1* activities in rats administered brewers' rice indicated that brewers' rice may be associated with an antioxidant enzyme. Therefore, the effect of treatments with brewers' rice on the SOD, MDA, and NO activities in AOM-injected rats was examined. The decreased SOD levels in the group treated with AOM alone illustrated that the defense mechanism may have been overwhelmed to alleviate the amount of superoxide produced by the carcinogen. The observed effect may also be due to the impairment of antioxidant enzymes, which act as safeguards for cells during reactive oxygen species (ROS) detoxification[43]. This finding implies that the group treated with AOM alone, in which carcinogenesis was induced but no brewers' rice treatment was administered, exhibited a reduction in SOD activity associated with a decreased antioxidative capacity. The group treated with AOM alone presented an increased MDA level and subsequently, increased lipid peroxidation, which was evident by the accumulation of β-catenin. Taken together, these findings suggest that the increased SOD and decreased MDA formation observed in the groups treated with brewers' rice may be associated with a high total phenolic content and the bioactive compounds present in brewers' rice, as reported by Tan *et al*[13]. Overall, the data obtained in this study suggest that brewers' rice has the potential to increase SOD levels and reduce the activities of MDA and NO.

The transcriptional inhibition of β-catenin and NF-κB activities may lead to a suppression of colon cancer development, which implies that the observed effects can likely be attributed to the dietary compositions present in brewers' rice. Most studies have demonstrated the additive and/or synergistic effects of some phytochemicals and nutrients[44-46]. Therefore, in the current study, rather than isolated compounds, brewers' rice was administered to the rats. Results from our earlier study indicated that brewers' rice consisted of a phenolic antioxidant, phytic acid, vitamin E, and γ-oryzanol[13]. The synergistic/additive activities of these components in brewers' rice may contribute to a negative regulation of the Wnt and NF-κB signaling pathways to induce the phosphorylation and degradation of β-catenin and NF-κB expression, as observed in the present study. In addition to the effects observed in the Wnt and NF-κB signaling pathways, it is plausible that the bioactive constituents present in brewers' rice facilitates the modulation of *Nrf2* and *Nrf2*-regulated *HO-1* expression, which subsequently enhances the antioxidant enzyme to mediate oxidative stress in the carcinogen-treated brewers' rice-fed groups. In conclusion, this study provides clear evidence that brewers' rice offers great potential against colorectal cancer *via* the regulation of Nrf2 expression

and the inhibition of the Wnt and NF-κB signaling pathways. However, this study has been limited to the use of brewers' rice in male Sprague-Dawley rats, and the duration of the treatment was only twenty weeks. Therefore, further studies are warranted in long-term animal studies or human clinical trials to confirm these findings. Uncontrolled signaling through the wingless/Wnt pathway and overexpression of NF-κB have been reported to play crucial roles in the development of colorectal cancer. Nrf2 is responsible in the regulation of phase Ⅱ detoxification and antioxidant enzymes. Our findings showed that the dietary administration of 40% (w/w) brewers' rice modulated the Wnt signaling pathway. Feeding 20% (w/w) brewers' rice improved the antioxidant level, which indicated that brewers' rice can effectively enhance recovery from oxidative stress induced by AOM. Taken together, these results strongly imply the potential use of brewers' rice in future applications to combat oxidative stress and colon carcinogenesis.

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COMMENTS

Background

The deregulation of Wnt/β-catenin signaling and overexpression of nuclear factor-kappa B (NF-κB) has been associated with colorectal cancer. Studies have reported that natural products exert many beneficial health effects. Brewers' rice, known locally as temukut, consists of broken rice, rice bran, and rice germ, which is a waste product produced in the rice industry. Although previous studies have demonstrated the anti-colon cancer activity of brewers' rice, the molecular mechanisms underlying these effects have yet to be studied.

Research frontiers

The authors aimed to investigate the mechanistic action of brewers' rice in regulating the Wnt/NF-κB/Nrf2-signaling pathways and assess the antioxidant effect of these treatments during colon carcinogenesis in male Sprague-Dawley rats.

Innovations and breakthroughs

This is the first study demonstrating that brewers' rice inhibited colon carcinogenesis *via* the modulation of multiple signaling pathways. The transcriptional inhibition of β-catenin and NF-κB activities and the activation of *Nrf2* and *HO-1* may be associated with the synergistic/additive effects of bioactive constituents present in brewers' rice.

Applications

The authors hypothesize that brewers' rice may provide chemopreventive or chemotherapeutic effects against colorectal cancer *via* the regulation of multiple signaling pathways. These findings suggest that brewers' rice offers great potential against colorectal cancer *via* the regulation of Nrf2 expression and the inhibition of the Wnt/NF-κB signaling pathways.

Terminology

Uncontrolled Wnt signaling pathway and overexpression of NF-κB have been reported to play a vital role in the development of colorectal cancer. Nrf2 is a key element in the ARE-mediated transcriptional machinery and plays a critical role in the regulation of phase Ⅱ detoxification and antioxidant enzymes.

Peer-review

This is a very interesting study that attempts to elucidate the molecular mechanisms by which brewers' rice could be a potential anticancer agent. This study is well-written, and its findings contribute to the understandings of the mechanisms through which rice may be beneficial in anticancer activity.

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ORIGINAL ARTICLE

Basic Study

Upregulation of nemo-like kinase is an independent prognostic factor in colorectal cancer

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Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at fugang416@126.com. Participants gave informed consent for data sharing.

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Abstract

AIM: To investigate the expression and oncogenic role of nemo-like kinase (NLK) in colorectal cancer.

METHODS: Expression of NLK protein was assessed by immunohistochemistry in tissue specimens from 56 cases of normal colorectal mucosa, 51 cases of colorectal adenoma, and 712 cases of colorectal cancer. In addition, NLK expression was knocked down using a lentivirus carrying NLK small hairpin RNA in colorectal cancer cells. Cell viability methylthiazoletetrazolium assays, colony formation assays, flow cytometry cell cycle assays, Transwell migration assays, and gene expression assays were performed to explore its role on proliferation and migration of colorectal cancer.

RESULTS: Expression of NLK protein progressively increased in tissues from the normal mucosa through adenoma to various stages of colorectal cancer. Overexpression of NLK protein was associated with advanced tumor-lymph node-metastasis stages, poor differentiation, lymph node and distant metastases, and a higher recurrence rate of colorectal cancer ($P < 0.05$). Multivariate analyses showed that NLK expression was an independent prognostic factor to predict overall

survival (hazard ratio 2.57, 95% confidence interval: 1.66-3.98; $P < 0.001$) and disease-free survival (hazard ratio 1.96, 95% confidence interval: 1.40-2.74: $P < 0.001$) of colorectal cancer patients. Furthermore, knockdown of NLK expression in colorectal cancer cell lines reduced cell viability, colony formation, and migration, and arrested tumor cells at the G0/G1 phase of the cell cycle. At the gene level, knockdown of NLK expression inhibited matrix metalloproteinase-2 expression in colorectal cancer cells.

CONCLUSION: NLK overexpression is an independent prognostic factor in colorectal cancer and knockdown of NLK expression inhibits colorectal cancer progression and metastasis.

Key words: Colorectal cancer; Gene regulation; Nemo-like kinase; Prognosis

Core tip: Altered expression of nemo-like kinase (NLK) protein is associated with cancer development. This study systematically evaluated NLK expression in different stages of colorectal cancer (CRC) for association with CRC prognosis. NLK expression progressively increased from normal tissues through adenoma, stage Ⅰ, Ⅱ, and Ⅲ, to stage Ⅳ CRC. However, knockdown of NLK expression significantly inhibited CRC cell growth, migration, cell cycle progression, and matrix metalloproteinase-2 expression. These data demonstrate that NLK overexpression is an independent CRC prognostic indicator and that knockdown of NLK expression inhibits CRC cell progression and metastasis.

Zhang W, He J, Du Y, Gao XH, Liu Y, Liu QZ, Chang WJ, Cao GW, Fu CG. Upregulation of nemo-like kinase is an independent prognostic factor in colorectal cancer. *World J Gastroenterol* 2015; 21(29): 8836-8847 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i29/8836.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i29.8836

INTRODUCTION

Colorectal cancer (CRC) remains a significant worldwide health problem and was responsible for more than 1.2 million new cancer cases and over 600000 cancer-related deaths in 2008 worldwide[1]. Currently, the tumor-lymph node-metastasis (TNM) staging system is utilized to stage the disease, guide treatment selections, and predict the prognosis for CRC patients[2,3]. However, utilization of this staging system is not always possible, such as in CRC detected by colonoscopy or malignant colorectal polyps resected under endoscopy[4]. In this regard, biologic markers could help us to precisely predict the prognosis, treatment responses, or even treatment selections (such as targeted therapy)[5-7]; in addition, it is easy to assess a small amount of tumor tissues[4]. Such an approach has been used to predict the long-term survival of CRC patients[8]. To date, due to the advancement of surgical techniques and the integration of chemoradiotherapy and targeted therapy, the prognosis of CRC patients has been significantly improved[9,10]. However, in advanced CRC patients and those with local recurrence or distant metastasis, the prognosis is still very poor[11,12]. Therefore, the molecular mechanisms and key regulators of CRC progression and metastasis need to be investigated to provide biomarkers to predict the risk of developing CRC local recurrence and distant metastasis[13], which could in turn enable us to optimally select treatment strategies and eventually improve patient prognosis.

Nemo-like kinase (NLK), an evolutionarily conserved serine/threonine protein kinase[14], regulates many transcription factors and signaling pathways that are important for determining cell fate[15,16]. NLK is an important regulator of several signal transduction pathways including Wnt and Notch signaling pathways, both of which play critical roles in tumorigenesis. NLK is able to regulate the Wnt/β-catenin signaling pathway by phosphorylation of lymphoid enhancer-binding factor 1 in neural progenitor cells[15]. In addition, NLK, as a negative regulator of the Notch signaling pathway, is able to inhibit formation of the transcriptionally active ternary complex of the notch intracellular domain, CSL, and mastermind[16]. Altered NLK expression also is associated with the development and progression of several human cancers[17-20]. Upregulation of NLK protein occurs in hepatocellular carcinoma[17], whereas NLK expression is downregulated in prostate cancer cells[18]. In other human cancers, knockdown of NLK expression was able to reduce tumor cell viability[19,20]. Furthermore, NLK gene variations are associated with ovarian cancer risk[21]. Because NLK is a member of the mitogen-activated protein kinase family, which functions to promote cell proliferation, in this study, we first evaluated the NLK expression level *via* immunohistochemistry in normal mucosa, adenoma, and CRC tissue specimens from 712 cases. We also included 16 familial adenomatous polyposis (FAP) patients and 21 metastatic CRC patients. Next, we knocked down NLK expression using a lentivirus carrying NLK small hairpin RNA (shRNA) to assess the effects on CRC cells, including cell viability, cell cycle distribution, colony formation, migration, and gene expression.

MATERIALS AND METHODS

Patients and tissue samples

The study population has been described previously[22]. Specifically, there were five groups of patients enrolled in this study. Group A consisted of 56 patients with normal rectal mucosa. Samples were obtained from patients with severe mixed hemorrhoids who underwent

the procedure for prolapse and hemorrhoids. All patients had morphologically normal colorectal mucosa that were free of neoplastic or inflammatory diseases and confirmed by preoperative colonoscopy[22]. Group B included 51 patients with colorectal adenomatous polyps. Group C included 742 patients with sporadic histologically confirmed CRC, including 53 stage Ⅰ, 312 stage Ⅱ, 322 stage Ⅲ, and 55 stage Ⅳ patients. Group D consisted of 16 FAP patients with concomitant CRC, each with a set of three matched specimens (normal mucosa, adenoma, and carcinoma). Group E consisted of 21 patients with metastatic CRC and concurrently resected metastatic carcinoma. Each of these metastatic patients also had a set of three matched specimens (normal mucosa and primary and metastatic tumors). Of these 21 metastatic carcinomas, 19 had liver metastases and 2 had greater omental metastases. All patients underwent surgical treatment in the Department of Colorectal Surgery, Changhai Hospital, The Second Military Medical University (Shanghai, China) between December 1999 and December 2009. All diagnoses were confirmed histopathologically by two independent pathologists. This study was approved by the Ethics Committee of Changhai Hospital, and written informed consent was obtained from all patients.

Tissue specimens were processed and embedded in paraffin, and paraffin blocks from each lesion were used to construct the tissue microarrays (TMAs) as described previously[22]. A total of six TMA blocks covering all the tissue specimens were constructed and used for immunostaining of NLK expression.

Immunohistochemistry

Immunohistochemistry was performed as described previously[22]. Briefly, the deparaffinized sections were incubated with 0.3% hydrogen peroxide and then with 20% goat serum to block nonspecific binding. Next, the TMA sections were incubated with a monoclonal anti-NLK antibody (ab69933; Abcam, Cambridge, United Kingdom) at a dilution of 1:100 for 2 h in a humidified chamber at room temperature. After that, the sections were further incubated with a secondary antibody and underwent a color reaction.

All immunostained TMA sections were reviewed and scored by two investigators (Gao XH and He J) who were blinded to the clinical information. The concordance rate was high (> 94%) and any disagreements were resolved by consensus. The level of NLK expression was scored using the criteria available on the ATLAS web site[23] and as described previously[22]. In particular, immunostaining of NLK protein was scored using the multiplication of the intensity and percentage of staining with a scale from 0 to 12. Staining of tumor cells with final staining scores of 0, 1-4, 5-8, and 9-12 was assigned as negative (-), slightly positive (+), moderately positive (++), and strongly positive (+++), respectively[22]. The scores were further categorized into lower expression (- to +) and higher expression (++ to +++) groups for analyses.

Cell lines and culture

An embryonic kidney HEK293T cell line and CRC SW480, SW620, RKO, DLD-1, HCT116, and HT-29 cell lines were obtained from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science (Shanghai, China) and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 ℃ in a humidified incubator with 5% CO_2.

Lentivirus packaging and cell infection

According to a previous protocol[20], we selected the NLK sequence (5′-GATAGACCTATTGGATATG-3′) according to GenBank data (NM_016231) to knock down NLK expression, and the negative control sequence used was 5′-TTCTCCGAACGTGTCACGT-3′. The double-strand oligonucleotides were synthesized and then cloned into the pFH-L vector (Shanghai Preii, Shanghai, China). Next, the lentivirus was generated, packaged, and used to infect cells according to the manufacturer's instructions. In brief, 293T cells were cotransfected with shRNA-expressing plasmids and the two helper plasmids pCMVΔR8.92 and pVSVG-I (Shanghai Preii) using Lipofectamine 2000 (Invitrogen of Thermo Fisher Scientific, Waltham, MA, United States). After 48 h, culture medium containing the packaged lentivirus was harvested and concentrated. To infect cells, HT-29 cells (5×10^4/well) were infected with lentivirus carrying NLK (Lv-shNLK) or negative control (Lv-shCon) shRNA with a multiplicity of infection of 50 using 2 μL of Polybrene (at a stock of 4 μg/μL) in 1 mL of virus/media at a final concentration of 8 μg/mL; 24 h later, the culture medium was replaced with a regular medium. The NLK knockdown efficiency was validated by quantitative real-time reverse transcription (qRT)-PCR and Western blotting 5 d after lentivirus infection. After confirming the knockdown efficiency, cells were seeded into 96-well plates for the methylthiazoletetrazolium (MTT) cell proliferation assay and 6-well plates for the colony formation assay and cell cycle analysis.

RNA isolation and qRT-PCR

Total cellular RNA was isolated using Trizol reagent (Invitrogen) and reversely transcribed into cDNA using M-MLV-RTase (Promega, Madison, WI, United States) according to the manufacturers' instructions. cDNA samples were then used for qPCR amplification of NLK using the SYBR-Green Master PCR Mix (Applied Biosystems of Thermo Fisher Scientific) in triplicate. qPCR amplification and data collection were performed on the TP800 qPCR System (Takara Bio Inc., Otsu, Shiga, Japan). All data were normalized to an endogenous control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The relative

value of NLK mRNA expression compared to the control was expressed as $2^{-(Ct-Cc)}$ (Ct and Cc were the mean threshold cycle differences after normalizing to GAPDH). The primers used for qPCR were as follows: GAPDH, 5′-TGACTTCAACAGCGACACCCA-3′ and 5′-GGAGTGTTGGAGAAGTCATATTAC-3′; and NLK, 5′-ATCATCAGCACTCGCATCATC-3′ and 5′-GACCAGACAACACCAAAGGC-3′.

Protein extraction and Western blot

Total cellular protein was extracted using a lysis buffer containing 100 mmol/L Tris, 4% sodium dodecyl sulfate (SDS), 10% glycerol, 200 mmol/L NaCl, and 2 mmol/L EDTA, and then quantified. For Western blot, cell lysates were separated in 12% SDS-polyacrylamide gels and transferred onto polyvinylidene fluoride membranes (Millipore Corp., Billerica, MA, United States). The membranes were then blocked in a 5% skim milk solution, followed by incubation in milk containing mouse anti-NLK monoclonal antibody (Abcam). Western blotting was developed using a horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Dallas, TX, United States) and was detected by an enhanced chemiluminescence reagent (Santa Cruz Biotechnology). GAPDH was used as an internal control for Western blotting analysis.

Cell viability MTT assay

Cells infected with Lv-shNLK or Lv-shCon were seeded in 96-well plates at a density of 2000 cells per well. At the indicated time points, 20 μL of MTT solution (5 mg/mL) was added to each well. The plates were further incubated for 4 h at 37 ℃, and 150 μL of dimethyl sulfoxide was added into each well to dissolve the crystals. After incubation for 10 min at room temperature, the absorbance was recorded at 490 nm.

Colony formation assay

CRC HT-29 cells were infected with Lv-shNLK or Lv-shCon. Five days later, the cells were collected and reseeded at 300 cells per well in 6-well plates in triplicate. The cells were incubated at 37 ℃ in 5% CO_2, and the growth medium was renewed every 3 d. After 14 d of culture, the plates were stained with Giemsa, and the numbers of colonies were counted and recorded.

Flow cytometry cell cycle assay

HT-29 cells infected with Lv-shNLK or Lv-shCon were inoculated in a 6-cm dish and cultured for 40 h. At the end of the experiments, 1×10^6 cells from each well were harvested and fixed in 70% ethanol for 1 h. After washing three times with ice-cold PBS, the cells were treated with 50 μL/mL propidium iodide solution (Sigma-Aldrich, St. Louis, MO, United States) and 100 μL/mL RNase in PBS for 15 min at room temperature in the dark and analyzed by flow cytometry (BD FACS Calibur; BD Biosciences, San Jose, CA, United States) according to the manufacturers' instructions.

Tumor cell Transwell migration assay

The Transwell migration assay was conducted as described previously[24]. Briefly, 2×10^4 parental and Lv-shCon- or Lv-shNLK-infected HT-29 cells were suspended in 200 μL of DMEM without FBS and placed into the upper chamber of the Transwells. The lower chamber was filled with 500 μL of DMEM containing 10% FBS. The cells were allowed to grow and migrate through these polycarbonate membranes with 8.0-μm-sized pores (Corning Inc., Corning, NY, United States) at 37 ℃ with 5% CO_2 for 24 h. Cells remaining on the upper surface of the filter were removed, and those that had migrated to the lower compartment were fixed with methanol, stained with crystal violet, and counted visually in five random fields under a light microscope. In addition, the migrated cells were dissociated, lysed, and quantified using a spectrophotometer at 570 nm. All of the experiments were performed in triplicate and repeated three times.

Statistical analysis

Associations between NLK expression and clinicopathologic variables were assessed by nonparametric (Mann-Whitney *U* or Kruskal-Wallis) tests. NLK expression in the paired tissue specimens, such as FAP and metastatic CRC, was compared using a paired nonparametric test (Wilcoxon test). Disease-free survival (DFS) was defined as the period from the date of surgery to the date of confirmed tumor relapse for relapsed patients or to the date of the last follow-up for non-recurrent patients. The Kaplan-Meier method was used to estimate the overall survival (OS) and DFS, and analyzed using the log-rank test. Cox proportional hazards models were used to estimate the survival distributions and hazard ratios. Data on the *in vitro* experiments were compared using the Student's *t* test. All statistical analyses were two-sided and conducted using SPSS software version 18.0 (SPSS Inc., Chicago, IL, United States). A $P < 0.05$ was considered as statistically significant.

RESULTS

Upregulated NLK expression from normal mucosa through adenoma to CRC ex vivo

TMAs containing CRC tissue specimens from 742 cases from our previous study[22] were used. During cutting, tissue samples from 30 cases were lost, leading to 712 CRC samples for the current study.

Immunostaining data showed that NLK was primarily expressed in the cytoplasm of epithelial cells and was significantly upregulated from the normal mucosa through adenoma to CRC (Figure 1). There were significant differences in NLK expression among these six groups of tissue specimens ($P < 0.001$; Table

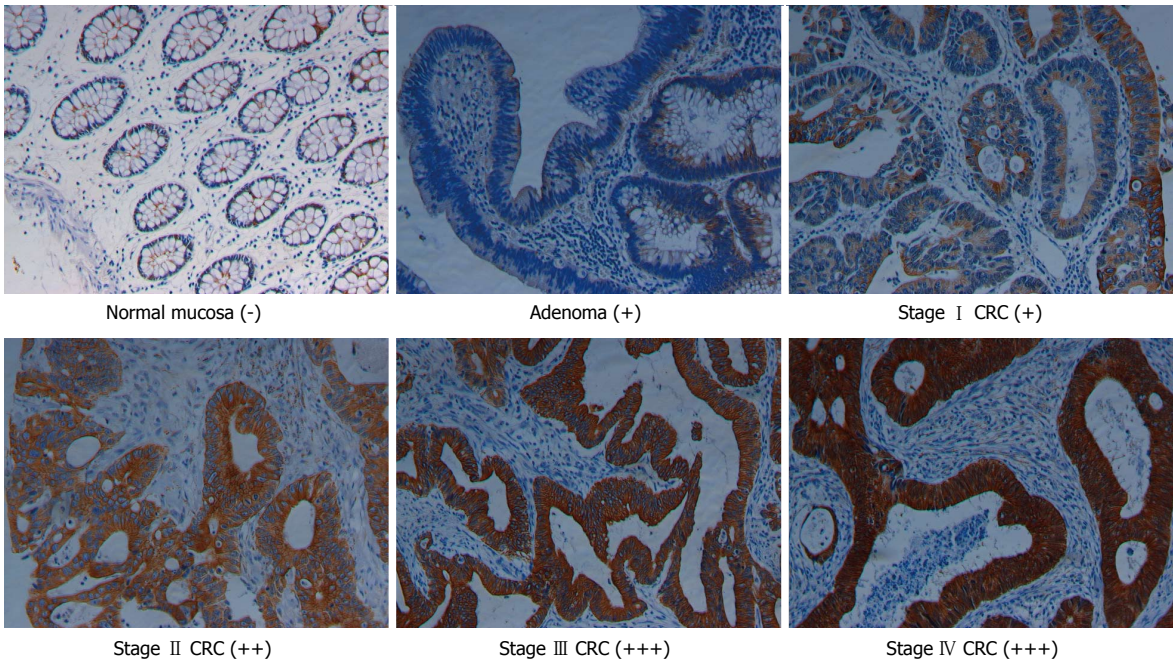

Figure 1 Representative immunohistochemical data of nemo-like kinase expression. Expression of NLK protein was mainly present in the cytoplasm of tumor cells, as indicated by the brown color (magnification × 200). CRC: Colorectal cancer.

Table 1 Differential expression of nemo-like kinase protein in normal mucosa, adenoma, and various stages of colorectal cancer tissue specimens

Characteristic	Total	NLK expression				Positive rate	Mean rank[1]	*P* value[2]
		-	+	+ +	+ + +			
Normal mucosa	56	14	35	6	1	75.0%	216.32	-
Adenoma	51	7	31	13	0	86.3%	273.63	0.049[a]
Stage Ⅰ	53	4	22	21	6	92.5%	392.97	0.005[c]
Stage Ⅱ	312	31	97	154	30	90.1%	410.66	0.573
Stage Ⅲ	298	24	76	165	33	91.9%	441.42	0.074
Stage Ⅳ	49	1	7	17	24	98.0%	596.47	< 0.001[b]

[1]Kruskal–Wallis test was used for the overall comparison ($P < 0.001$); [2]Mann-Whitney U test was used to determine significant differences between two groups. [a]$P \leqslant 0.05$ *vs* normal mucosa; [c]$P \leqslant 0.05$ *vs* adenoma; [b]$P \leqslant 0.01$ *vs* stage Ⅲ cancer.

1). Furthermore, overexpression of NLK protein in the tumor tissues was verified using Western blot, and overexpression of NLK mRNA was verified using qRT-PCR with ten cases of CRC and paired normal mucosae (Supplemental materials). The level of NLK mRNA in the tumor tissues was significantly higher than in the normal tissues ($P < 0.05$).

Next, we analyzed NLK expression in mucosa, adenoma, and adenocarcinoma tissues from 16 FAP patients. These data showed that NLK expression was significantly increased between normal mucosa and adenoma tissues ($P < 0.001$), as well as between adenoma and adenocarcinoma tissues ($P = 0.003$; Figure 2).

We also analyzed the NLK expression in normal mucosa as well as primary and metastatic CRC tissues from 21 patients. We found that the NLK expression in primary CRC was significantly higher than in the normal mucosa ($P = 0.024$), but there was no significant difference between metastatic and primary tumors (Figure 3).

Association between NLK expression and clinicopathologic factors

The clinicopathologic variables are shown in Table 2. In brief, overexpression of NLK protein was significantly associated with tumor lymph node metastases, distant metastasis, advanced TNM stages, poorer tumor differentiation, and higher recurrence rate (all $P < 0.05$). Moreover, NLK expression was significantly higher in rectal cancer than in colon cancer ($P < 0.001$). There was no significant association between NLK expression and other clinicopathologic factors.

Association of NLK expression with survival of CRC patients

Patients with NLK-overexpressing tumors had a worse OS and DFS compared to those without or with weakly NLK-expressing tumors (all $P < 0.001$; Figure 4). Univariate analyses identified advanced TNM stages, colon cancer, elevated serum carcinoembryonic antigen (CEA), elevated serum cancer antigen (CA)19-9, overexpression of NLK protein, and postoperative radiochemotherapy as associated with a shorter OS (all $P < 0.01$), whereas advanced TNM stages, elevated serum CEA, elevated serum CA19-9, higher expression of NLK, and postoperative radiochemotherapy were associated with a shorter DFS (all $P < 0.001$) (Table 3). Multivariate analysis showed that advanced TNM stages, poor tumor differentiation, rectal cancer, elevated serum CEA, and higher expression of NLK

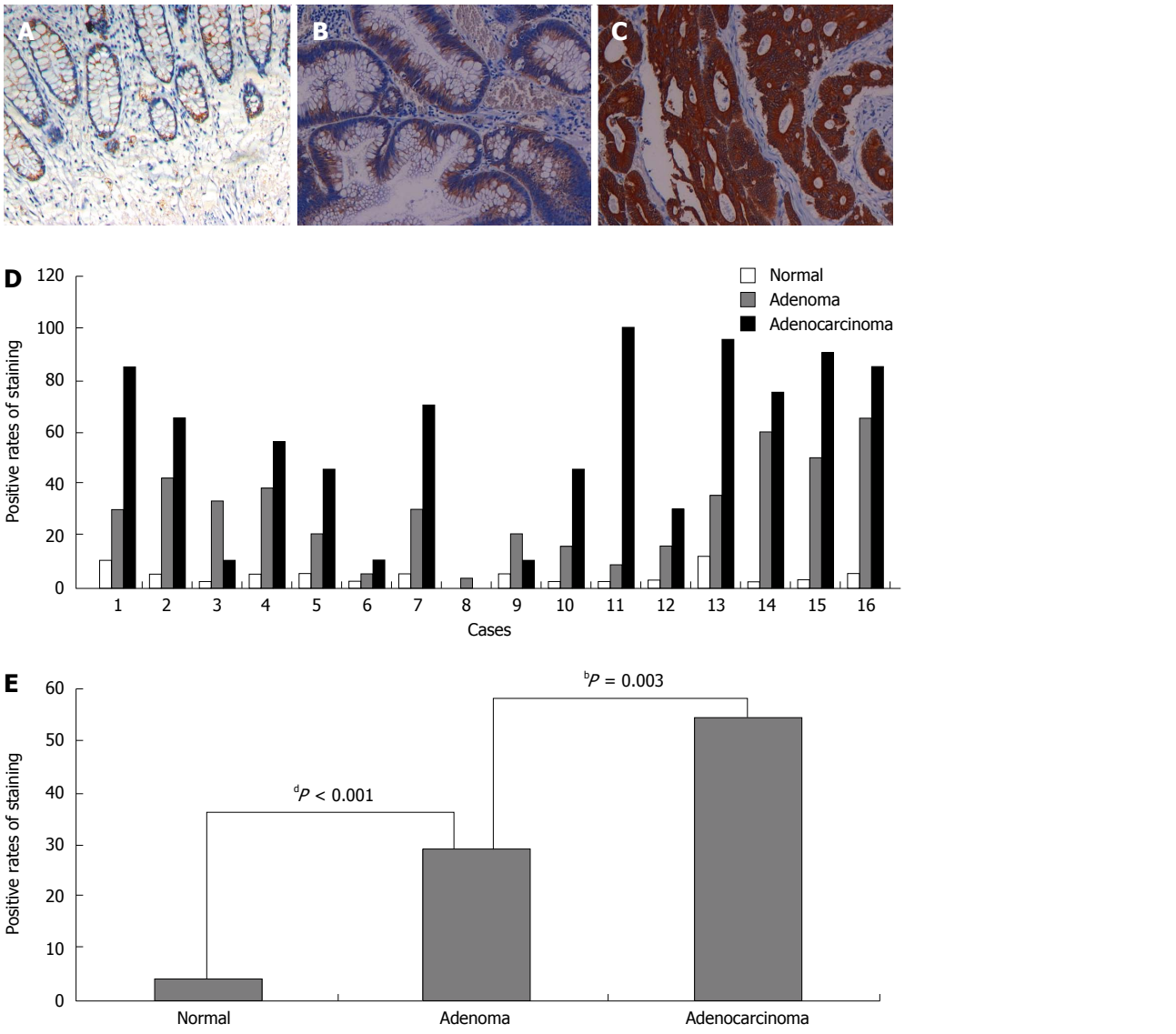

Figure 2 Immunohistochemical detection of nemo-like kinase expression in familial adenomatous polyposis. Representative sections of A: Normal mucosa; B: Adenoma; and C: Adenocarcinoma; D: Individual; and E: Pooled quantification of staining from 16 familial adenomatous polyposis patients. $^{b}P < 0.01$, $^{d}P < 0.001$ *via* Wilcoxon test.

were independent predictive factors for a shorter OS (all $P < 0.05$), whereas advanced TNM stage, elevated serum CA19-9, and overexpression of NLK protein were independent predictive factors for a shorter DFS (all $P < 0.01$).

Effects of NLK knockdown on tumor cell viability, colony formation, and migration

To explore the role of NLK in CRC, we first detected NLK expression in six different colorectal cell lines using qRT-PCR (Figure 5A). These data showed that NLK was expressed in all cell lines, with the highest NLK level in HT-29 cells. Thus, we selected HT-29 cells for our knockdown experiments. Specifically, NLK expression was downregulated by Lv-shNLK compared to Lv-shCon. At the highest infection efficiency, green fluorescent protein was expressed in > 90% of infected HT-29 cells. Five days after infection, the levels of NLK mRNA and protein were reduced significantly in HT-29 cells infected with Lv-shNLK compared to Lv-shCon ($P < 0.05$; Figure 5B and C).

Next, we assessed the effects of NLK knockdown on regulation of tumor cell viability and colony formation. We found that knockdown of NLK expression reduced the numbers of HT-29 cells compared to the control group ($P < 0.05$; Figure 5D). The colony formation assay also showed that NLK knockdown in HT-29 cells resulted in fewer colonies compared to the control group ($P < 0.05$; Figure 5E). The Transwell tumor cell migration assay showed that a significantly lower proportion of HT-29 cells with NLK knockdown was able to migrate into the bottom chambers compared to the control group ($P < 0.01$; Figure 5F).

Effects of NLK knockdown on regulation of tumor cell cycle progression

A flow cytometric cell cycle assay was performed using HT-29 cells 5 d after infection. These data showed

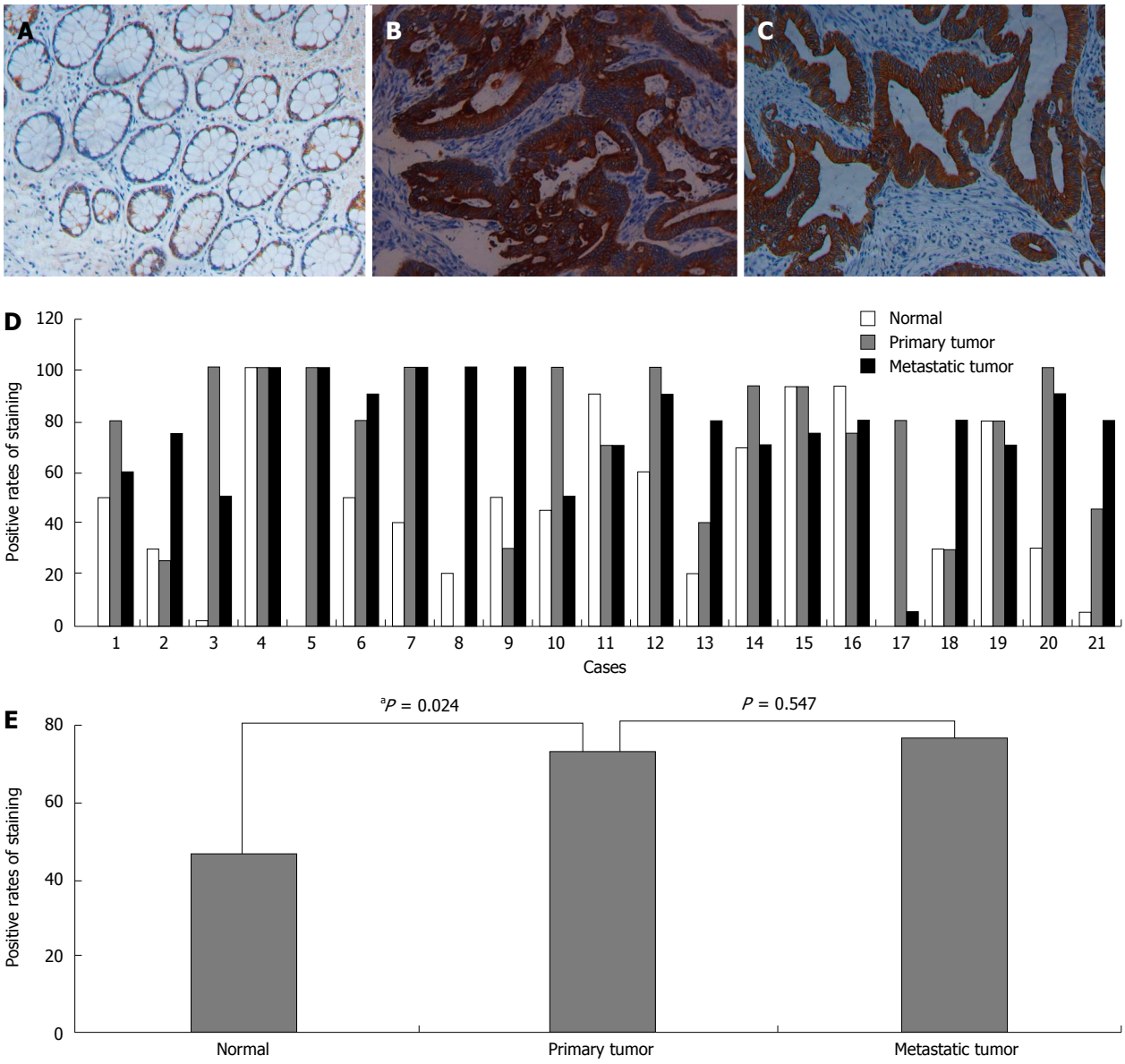

Figure 3 Immunohistochemical detection of nemo-like kinase expression in colorectal cancer. Representative sections of A: Normal mucosa; B: Primary tumor; C: Metastatic tumor; D: Individual; and E: Pooled quantification of staining from 21 patients with metastatic colorectal cancer. $^{a}P < 0.05$ *via* Wilcoxon test.

that after HT-29 cells were infected with Lv-shNLK, the number of G0/G1 phase cells was increased significantly ($P < 0.01$), whereas the number of S phase cells was reduced significantly ($P < 0.01$), indicating that Lv-shNLK infection arrested HT-29 cells at the G0/G1 phase of the cell cycle (Figure 6A and B). NLK knockdown also reduced the levels of matrix metalloproteinase-2 mRNA and protein (both $P < 0.01$; Figure 6C and D).

DISCUSSION

To date, numerous studies have reported an association between NLK expression and cancer development[25]. In the current study, NLK expression was analyzed in five groups of colorectal tissue specimens and then *in vitro* experiments were performed to assess the potential role of NLK in CRC development and progression. The results show that NLK protein is progressively overexpressed from the normal mucosa through adenoma to various stages of CRC, and that NLK overexpression is associated with advanced tumor TNM stages, poor differentiation, lymph node and distant metastases, and a higher recurrence rate of CRC. NLK expression was also one of the independent prognostic factors to predict OS and DFS of CRC patients. These results are consistent with the those reported by Chen *et al*[26] who showed that patients with NLK-positive tumors had higher rates of recurrence and mortality than patients with NLK-negative tumors, and that NLK expression was an independent factor of OS and DFS in CRC patients[26]. However, Han *et al*[27] found different results based on expression of NLK mRNA in 92 CRC cases. This inconsistency may be explained by the difference between expression of NLK protein and mRNA. In addition, the *in vitro* data from the current

Table 2 Association of nemo-like kinase expression with clinicopathologic parameters of colorectal cancer patients (*n* = 712)

Parameter	NLK immunostaining				*P* value
	-	+	+ +	+ + +	
Sex					0.165[1]
Male	29	112	210	55	
Female	31	90	147	38	
Age (yr)					0.408[1]
< 60	33	129	211	65	
⩾ 60	27	73	146	28	
Tumor location					< 0.001[1]
Colon cancer	48	110	145	36	
Rectal cancer	12	92	212	57	
Invasion depth					0.980[1]
T1-T2	6	27	42	12	
T3-T4	54	175	315	81	
Lymph node metastasis					0.027[2]
N0	35	122	180	43	
N1	21	54	120	36	
N2	4	26	57	14	
Distant metastasis					< 0.001[1]
M0	59	195	340	69	
M1	1	7	17	24	
TNM stage					< 0.001[2]
Ⅰ	4	22	21	6	
Ⅱ	31	97	154	30	
Ⅲ	24	76	165	33	
Ⅳ	1	7	17	24	
Tumor differentiation					0.019[1]
Well, moderate	42	182	326	82	
Poor, mucinous	18	20	31	11	
Serum CEA (ng/mL)					0.570[1]
< 5	39	123	220	63	
⩾ 5	21	79	137	30	
Serum CA19-9 (U/mL)					0.264[1]
< 37	51	178	294	79	
⩾ 37	9	24	63	14	
Recurrence					< 0.001[1]
No	56	176	257	68	
Yes	4	26	80	25	

[1]Mann-Whitney *U* test; [2]Kruskal-Wallis test. CA19-9: Cancer antigen 19-9; CEA: Carcinoembryonic antigen; TNM: Tumor-node-metastasis.

study also support the *ex vivo* results; for example, knockdown of NLK expression *in vitro* reduced CRC cell viability, colony formation, and migration, and arrested tumor cells at the G0/G1 phase of the cell cycle. Knockdown of NLK expression also inhibited matrix metalloproteinase-2 expression in CRC cells. Taken together, results from the current study support the notion that NLK overexpression plays an important role in CRC development and progression.

TMA technology[28] allows for analysis of an entire cohort of tissue samples in one batch of experiments, which eliminates the staining bias of multiple day experiments[29]. However, it may produce sample selection bias. Our data demonstrate that NLK protein is overexpressed in CRC, but not in normal mucosae, with intermediate expression in adenoma tissues. Overexpression of NLK protein was associated with poorer clinicopathologic parameters, such as an advanced TNM stage, poor differentiation, lymph node and distant metastases, and a higher recurrence rate in CRC, thus contributing to poor OS and DFS of these CRC patients. These data are consistent with the overexpression of NLK in other organ sites of cancer[7]. Indeed, NLK is a member of the mitogen-activated protein kinase family that functions to promote cell proliferation; thus, overexpression of NLK could lead to cell proliferation and malignant transformation. Furthermore, the multivariate analysis in this study confirmed that NLK expression is one of the independent prognostic factors of the OS and DFS for CRC patients.

One unique feature of this study is that over-expression of NLK was confirmed in the paired tissues of normal mucosa, adenoma, and adenocarcinoma from 16 FAP patients, as well as in the paired tissues of normal mucosa and primary and metastatic CRC tissues from 21 patients. In general, hereditary heterogeneity produces variable gene expression in different tissues; however, evaluation of tissues from the same patient at different developing stages of CRC can minimize such heterogeneity. The data presented here further support the notion that NLK overexpression by cancer cells plays an oncogenic role in transformation of normal mucosa through adenoma to adenocarcinoma. NLK expression was not significantly different between primary and secondary tumor tissues, but the overall data show that NLK overexpression is associated with CRC lymph node and distant metastases, suggesting that NLK overexpression could induce tumor metastasis.

To assess the effects of NLK knockdown on tumor cell phenotypes *in vitro*, this study utilized shRNA-carrying lentiviruses, which effectively silence gene expression *in vitro* and *in vivo*[20]. The data show that knockdown of NLK expression significantly reduces HT-29 cell viability, colony formation, and migration capacity. These *in vitro* data further support the *in vivo* data, suggesting that NLK may play an oncogenic role in CRC. Therefore, NLK might serve as a potential therapeutic target for the future control of CRC. However, a previous study by Yasuda *et al*[30] showed that overexpression of NLK is associated with a decrease in tumor cell growth and induced apoptosis of the human colon cancer cell line DLD-1. Their data suggest that NLK might function as a tumor suppressor in colon cancer, which is contrary to the current findings. The reason for this discrepancy is unknown; but in their study, induction of wild-type NLK induced growth suppression of DLD-1 cells, whereas the kinase-negative mutant did not have such an effect, indicating that NLK may not function as a tumor suppressor in DLD-1 cells. The data of the current study also confirmed this finding, further suggesting that NLK does not play any role in DLD-1 tumorigenesis or maintenance of tumor phenotypes. In this case, NLK overexpression or an NLK kinase-

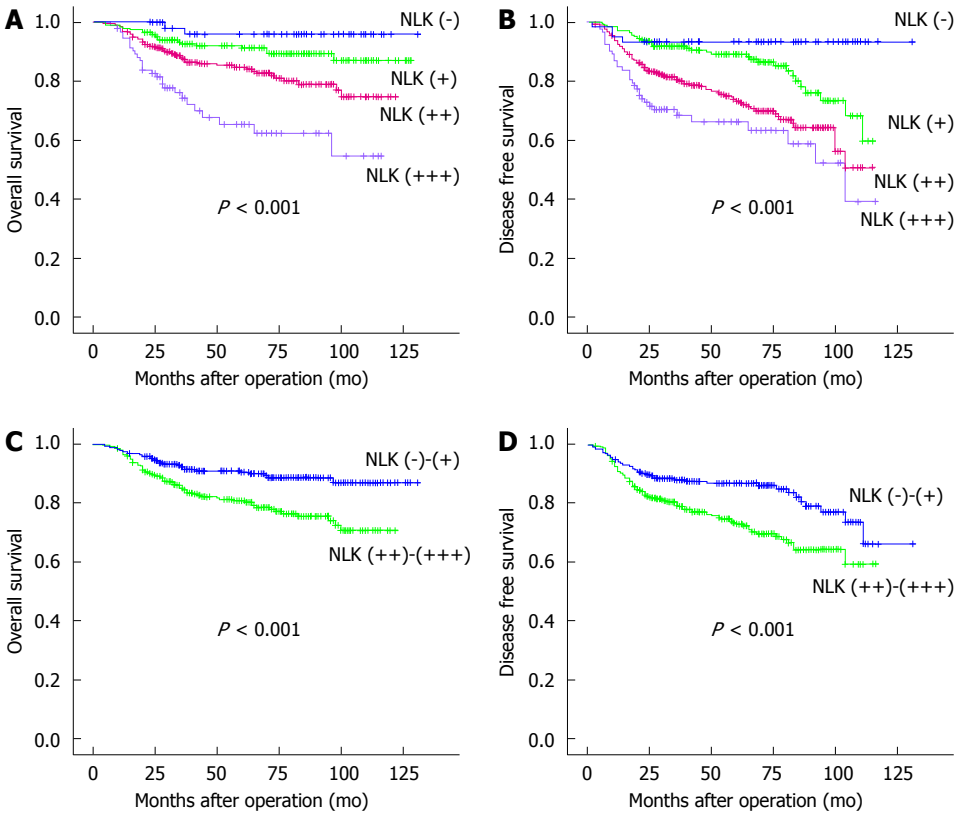

Figure 4 Kaplan-Meier survival curves. A,C: Overall survival; B,D: Disease-free survival stratified by nemo-like kinase (NLK) expression in 712 colorectal cancer patients.

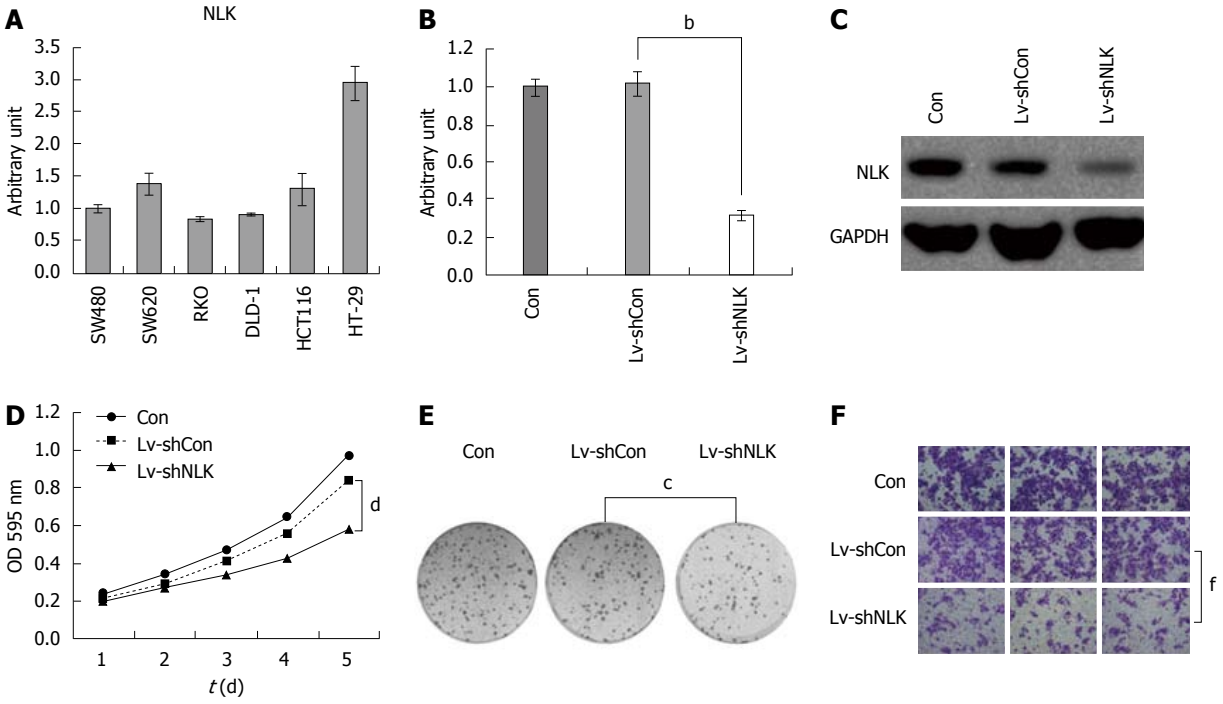

Figure 5 Knockdown of nemo-like kinase expression affects viability, colony formation, and migration of colorectal cancer cells. A: Nemo-like kinase (NLK) mRNA expression was assessed in six CRC cell lines using quantitative real-time-PCR; Knockdown of NLK B: mRNA and C: Protein expression in HT-29 cells 48 h after infection with a lentivirus carrying NLK (Lv-shNLK) or control (Lv-shCon) shRNA; D: Cell viability MTT assay; E: Colony formation assay at 14 d after infection; F: Transwell tumor cell migration assay. $^{b}P < 0.01$, $^{c}P < 0.05$; $^{d}P < 0.01$; $^{f}P < 0.01$.

Table 3 Univariate and multivariate analyses in colorectal cancer tissue specimens (n = 712)

Variables	Overall survival		Disease-free survival	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Univariate analyses				
Sex (male *vs* female)	0.84 (0.58-1.23)	0.376	0.86 (0.63-1.18)	0.342
Age (≥ 60 yr *vs* < 60 yr)	1.24 (0.85-1.80)	0.259	0.95 (0.69-1.30)	0.734
TNM stage (Ⅳ/Ⅲ *vs* Ⅱ/Ⅰ)	3.11 (2.06-4.70)	< 0.001	2.37 (1.72-3.27)	< 0.001
Differentiation (poor/mucinous adenocarcinoma *vs* well/moderate)	1.54 (0.93-2.55)	0.094	1.33 (0.85-2.08)	0.219
Position (rectal *vs* colon)	0.56 (0.38-0.83)	0.003	0.92 (0.66-1.23)	0.506
CEA (≥ 5 ng/mL *vs* < 5 ng/mL)	1.97 (1.36-2.85)	< 0.001	1.63 (1.19-2.21)	0.002
CA19-9 (≥ 37 U/mL *vs* < 37 U/mL)	2.27 (1.50-3.42)	< 0.001	1.89 (1.31-2.72)	0.001
NLK (++/+++ *vs* -/+)	2.19 (1.44-3.35)	< 0.001	2.71 (1.86-3.94)	< 0.001
Postoperative radiochemotherapy (yes *vs* no)	2.42 (1.30-4.51)	0.005	2.69 (1.60-4.50)	< 0.001
Multivariate analyses				
TNM stage (Ⅳ/Ⅲ *vs* Ⅱ/Ⅰ)	3.02 (1.99-4.59)	< 0.001	2.34 (1.70-3.22)	< 0.001
Differentiation (poor/mucinous adenocarcinoma *vs* well/moderate)	1.78 (1.06-2.98)	0.029	1.45 (0.94-2.24)	0.091
Position (rectal *vs* colon)	0.49 (0.33-0.72)	< 0.001	0.80 (0.59-1.08)	0.143
CEA (≥ 5 ng/mL *vs* < 5 ng/mL)	1.56 (1.04-2.35)	0.032	1.31 (0.95-1.81)	0.099
CA19-9 (≥ 37 U/mL *vs* < 37 U/mL)	1.54 (0.99-2.41)	0.058	1.76 (1.24-2.49)	0.002
NLK (++/+++ *vs* -/+)	2.57 (1.66-3.98)	< 0.001	1.96 (1.40-2.74)	< 0.001
Postoperative radiochemotherapy (yes *vs* no)	1.04 (0.50-2.16)	0.921	1.64 (0.93-2.92)	0.090

CA19-9: Cancer antigen 19-9; CEA: Carcinoembryonic antigen; CI: Confidence interval; HR: Hazard ratio; NLK: Nemo-like kinase; TNM: Tumor-node-metastasis.

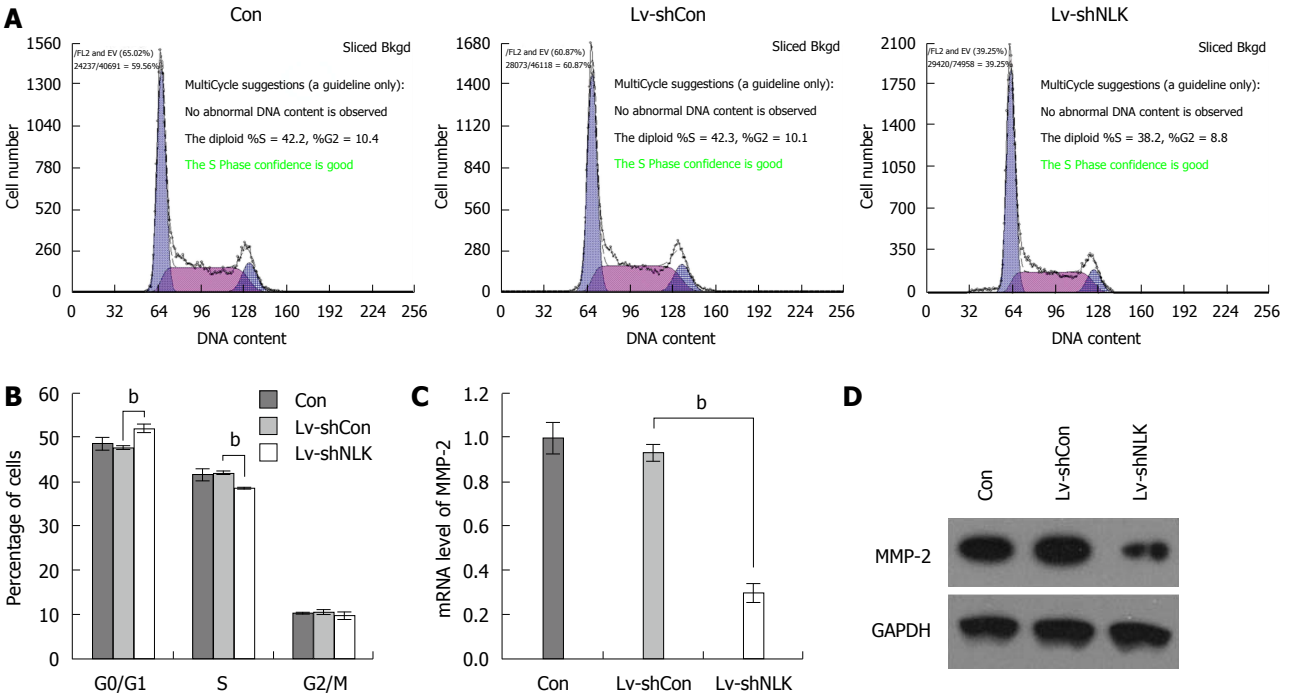

Figure 6 Knockdown of nemo-like kinase expression affects cell cycle progression and matrix metalloproteinase-2 expression in HT-29 cells. A: Flow cytometric cell cycle distribution in HT-29 cells 48 h after infection with a lentivirus carrying nemo-like kinase (NLK) (Lv-shNLK) or control (Lv-shCon) shRNA; B: Summarized data of cell cycle distribution; matrix metalloproteinase (MMP)-2; C: mRNA; and D: Protein expression with NLK knockdown. $^{b}P < 0.01$.

negative mutation may either have no effect on cell behavior or, in their case, NLK overexpression had tumor suppressive effects on CRC DLD-1 cells. CRC is a heterogeneous disease and different CRC cell lines have specific subgroup characteristics. Furthermore, the *ex vivo* data presented here further support our current hypothesis. In addition, NLK has been shown to exhibit dual and opposite effects in Wnt/β-catenin signaling in different *in vivo* situations[25]. A previous study showed that NLK might be involved as an oncogene in the tumorigenesis and progression of CRC[31]. Thus, further studies are needed to clarify this discrepancy and the overall role of NLK in CRC.

The current study has several limitations that should be addressed. For example, this study was retrospective, the patients were not randomly selected, and loss of follow-up could have introduced possible biases. Furthermore, the patients included in this

study did not receive neoadjuvant chemoradiotherapy, as the treatment would affect gene expression. Thus, the results obtained from this population may not generalize to the clinical setting. In addition, the TMA technology, like any technology, has certain limitations[32]. We chose this method for its reduced time, cost, and tissue samples needed[29]. Lastly, NLK knockdown was only performed in HT-29 cells, and more cell lines are needed to confirm the finding.

COMMENTS

Background

Nemo-like kinase (NLK) is an important regulator of several signal transduction pathways, including the Wnt and Notch signaling pathways, both of which play a critical role in tumorigenesis. Dysregulation of NLK expression was closely associated with progression of different human cancers.

Research frontiers

Altered NLK expression is associated with cancer development and progression; for example, upregulated NLK protein occurs in hepatocellular carcinoma, whereas NLK expression is downregulated in prostate cancer cells. In other human cancers, knockdown of NLK expression reduces tumor cell viability.

Innovations and breakthroughs

In this study, the authors systematically evaluated NLK expression in different developing stages of colorectal cancer (CRC) and investigated the prognostic value of NLK expression. We observed that NLK expression gradually increased in colorectal samples from normal tissues, adenoma, stage Ⅰ, stage Ⅱ, and stage Ⅲ, to stage Ⅳ CRC. Knockdown of NLK in CRC cells significantly inhibited cell growth, migration, cell cycle progression, and matrix metalloproteinase-2 expression. The data of this study suggest that NLK overexpression is an independent prognostic factor in CRC, and that knockdown of NLK expression inhibits CRC progression and metastasis.

Applications

The current study demonstrated that NLK overexpression plays an important role in CRC development and progression. This finding may help to understand the mechanism of CRC metastasis and to promote the prevention, diagnosis, and management for CRC patients.

Terminology

Tissue microarray technology has the advantage that the entire cohort of studied tissue samples in one batch of experiments. It has multiple advantages such as conserving reagents, saving time, and decreasing the amount of required tissue. However, the main limitation is sample selection bias, as the selected specimen may not be representative of the entire tissue.

Peer-review

This is an interesting paper assessing the role of NLK in CRC. The authors analyze a large series of clinical cases using TMA and correlate NLK expression with clinical parameters, and added some *in vitro* functional data of NLK knockdown to confirm the *ex vivo* data on the biomarker analysis. Their data indicated that NLK is a relevant molecule predicting CRC aggressiveness and prognosis.

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ORIGINAL ARTICLE

Basic Study

Influence of perfusate on liver viability during hypothermic machine perfusion

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Author contributions: Jia JJ and Zhang J contributed equally to this work; Zhou L, Li JH and Zheng SS conceived and designed the experiments; Chen XD and Xie HY contributed reagents/materials/analytic tools; Jia JJ and Zhang J wrote the manuscript; Jia JJ, Zhang J, Jiang L, Zhou YF and He N performed the experiments; Jia JJ and Zhang J analyzed the data.

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Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at shusenzheng@zju.edu.cn or surgeonlee@126.com

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Abstract

AIM: To optimize the perfusates used for hypothermic

machine perfusion (HMP).

METHODS: Sprague-Dawley rats were assigned randomly to three groups (n = 12 per group) that received either saline, University of Wisconsin cold-storage solution (UW) or histidine-tryptophan-ketoglutarate solution (HTK) as the perfusate. Each group was divided into two subgroups: static cold storage (SCS) and HMP (n = 6 per subgroup). The liver graft was retrieved according to the method described by Kamada. For the SCS group, the graft was directly placed into cold perfusate (0-4 ℃) for 6 h after liver isolation while the portal vein of the graft was connected to the perfusion machine for the HMP group. Then the perfusates were collected at different time points for analysis of aspartate aminotransferase (AST), alanine transaminase (ALT) and lactate dehydrogenase (LDH) levels. Liver tissues were obtained for evaluation of histology, dry/wet weight (D/W) ratio, and malondialdehyde (MDA) and adenosine-triphosphate (ATP) levels. The portal vein pressure and velocity were monitored in real time in all HMP subgroups.

RESULTS: Comparison of HMP and SCS: Regardless of the perfusate, HMP improved the architecture of donor graft in reducing the congestion around sinusoids and central vein and maintaining sinusoid lining in morphology; HMP improved liver function in terms of ALT, AST and LDH, especially during the 3-6 h period (SCS *vs* HMP using saline: ALT3, 225.00 ± 105.62 *vs* 49.50 ± 18.50, P = 0.047; LDH3, 1362.17 ± 563.30 *vs* 325.75 ± 147.43, P = 0.041; UW: LDH6, 2880.14 ± 948.46 *vs* 2135.00 ± 174.27, P = 0.049; HTK, AST6, 307.50 ± 52.95 *vs* 185.20 ± 20.46, P = 0.041); HMP decreased MDA level (saline, 2.79 ± 0.30 *vs* 1.09 ± 0.09, P = 0.008; UW, 3.01 ± 0.77 *vs* 1.23 ± 0.68, P = 0.005; HTK, 3.30 ± 0.52 *vs* 1.56 ± 0.22, P = 0.006). Comparison among HMP subgroups: HTK showed less portal vein resistance than UW and saline (*vs* saline, 3.41 ± 0.49 *vs* 5.00 ± 0.38, P < 0.001; *vs* UW, 3.41 ± 0.49 *vs* 4.52 ± 0.63, P = 0.007); UW reduced edema most efficiently (*vs* saline, 0.68 ± 0.02 *vs* 0.79 ± 0.05, P = 0.013),_while HTK maintained ATP levels best (*vs* saline, 622.60 ± 29.11 *vs* 327.43 ± 44.66, P < 0.001; *vs* UW, 622.60 ± 29.11 *vs* 301.80 ± 37.68, P < 0.001).

CONCLUSION: HMP is superior to SCS in maintaining both architecture and function of liver grafts. Further, HTK was found to be the optimal perfusate for HMP.

Key words: Hypothermic machine perfusion; Static cold storage; Liver viability; Wisconsin cold-storage solution; Histidine-tryptophan-ketoglutarate solution

Core tip: Although static cold storage (SCS) is the gold standard for liver transplantation, hypothermic machine perfusion (HMP) is currently challenging the limitations of SCS. However, there is no consensus on the basic setting for HMP, including the ideal perfusate. Here we compared the most common preservation solutions [University of Wisconsin cold-storage solution and histidine-tryptophan-ketoglutarate solution (HTK), saline as control] and found that HMP is superior to SCS regardless of different solutions and HTK seems to be the optimal perfusate for HMP.

Jia JJ, Zhang J, Li JH, Chen XD, Jiang L, Zhou YF, He N, Xie HY, Zhou L, Zheng SS. Influence of perfusate on liver viability during hypothermic machine perfusion. *World J Gastroenterol* 2015; 21(29): 8848-8857 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i29/8848.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i29.8848

INTRODUCTION

Static cold storage (SCS) is currently the gold standard method for preservation of the donor graft for liver transplantation (LT), with University of Wisconsin cold-storage solution (UW) and histidine-tryptophan-ketoglutarate solution (HTK) being the most commonly used preservation solutions[1]. The principal aim of SCS is to minimize preservation-related injury by rapidly cooling the graft core temperature to reduce metabolism[2]. Hypothermic machine perfusion (HMP) was first proposed by Belzer in the early 1960s, but the complex nature of this technique, the cumbersome equipment required, and lack of clarity regarding the mechanisms have limited its wide usage in clinical settings. However, in the era of the donor organ shortage, HMP is of increasing interest in transplantation centers because it allows better monitoring of the graft, improves washout of injurious waste products, and allows pharmaceutical intervention, leading to decreased ischemia reperfusion (I/R) injury which could be indirectly measured by malondialdehyde (MDA), and extended preservation, even in cases of resuscitated marginal organs or those obtained from cardiac death donors (DCD)[3,4]. Mounting evidence shows that HMP challenges the limitations of SCS in terms of reduced preservation-related injury and improved LT outcome[5-7]. However, there is no consensus on the basic conditions of HMP, including the optimal perfusate, pressure and flow velocity, double or single vessel perfusion (retrograde or antegrade, pulsatile or not), and with or without oxygen. In this study, we aimed to identify the optimal perfusate by comparison of the influence of different perfusates on liver viability during HMP.

MATERIALS AND METHODS

Experimental animals and groups

Adult male Sprague-Dawley rats (weight, 250-300 g) were used as the experimental animals. The rats were housed in a temperature-controlled environment

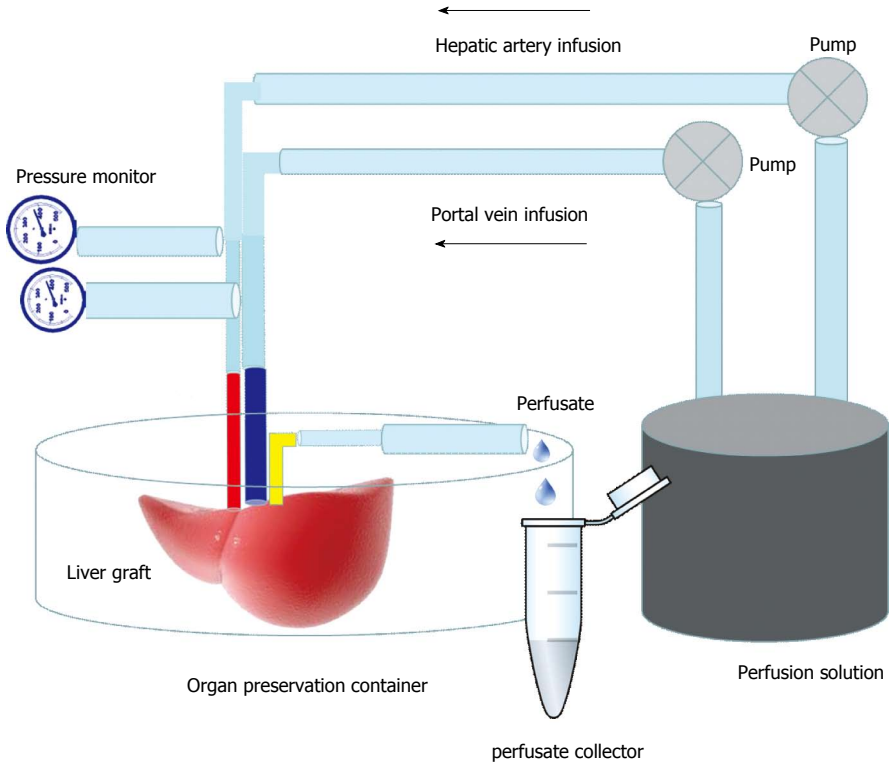

Figure 1 Schematic diagram of hypothermic machine perfusion. In this study, single vessel (portal vein) perfusion was performed.

(25-30 ℃) and provided with a standard diet with water *ad libitum*. Rats were assigned randomly to three groups (n = 12 per group) that received either saline, UW or HTK solutions as the perfusate. Each group was then divided into two subgroups: SCS and HMP (n = 6 per subgroup). All procedures used in this study were approved by the Ethics Committee for the Use of Experimental Animals of Zhejiang University (China) and were carried out in accordance with the ARRIVE (Animal Research: Reporting *in vivo* Experiments) guidelines (http://www.nc3rs.org/ARRIVE).

Surgical procedures and HMP conditions

The donor animal was anesthetized by intraperitoneal injection of 4% chloral hydrate (Shanghai No. 1 Biochemical and Pharmaceutical Company, China) and the liver graft was retrieved according to the method described by Kamada[8]. After the donor liver was isolated, the graft was perfused through the portal vein with cooled saline containing 25 U/mL heparin. In the SCS group, the graft was then placed into cold perfusate (0-4 ℃) for 6 h. In the HMP group, the portal vein of the graft was connected to the perfusion machine (Figure 1), which consisted of a BT200-2J low flow peristaltic pump (Xi'an Yima Opto-electrical Company, China), a four-channel physiological recorder (BL-420S, Biomart, China), a low-temperature thermostat (DC-1015, Shanghai Bilon Instrument Company, China) and a computer for 6 h. The perfusion machine settings were as follows: temperature, 4 ℃; portal vein velocity, 2 rpm (1.4 mL/min non-pulsatile delivery); perfusate volume, 60 mL. The pressure of the portal vein was monitored in real time and recorded using the computer.

Sample collection

At 0, 1, 3, and 6 h during the perfusion process, 2 mL perfusate samples were collected from each group for the analysis of liver function. At 6 h, liver tissues were obtained and fixed in 10% neutral formalin for later histological evaluation. The left lateral lobe was collected for determination of the dry/wet weight (D/W) ratio and other liver tissues were stored at -80 ℃ for further analysis.

Histopathologic examination and liver function tests

Excised liver specimens were fixed in 10% neutral buffered formalin for 48 h before being paraffin-embedded and sectioned (thickness, 4 μm) according to standard procedures. The sections were deparaffinized and hydrated gradually, and examined by hematoxylin and eosin staining. Morphological assessment was performed by an experienced liver pathologist in a blinded fashion. The levels of aspartate aminotransferase (AST), alanine transaminase (ALT) and lactate dehydrogenase (LDH) were analyzed with a Hitachi 7600 automatic analyzer (Hitachi, Tokyo, Japan).

Levels of lipid peroxidation and wet/dry weight ratio

Malondialdehyde (MDA) is considered to represent an indirect measurement of oxidative damage induced

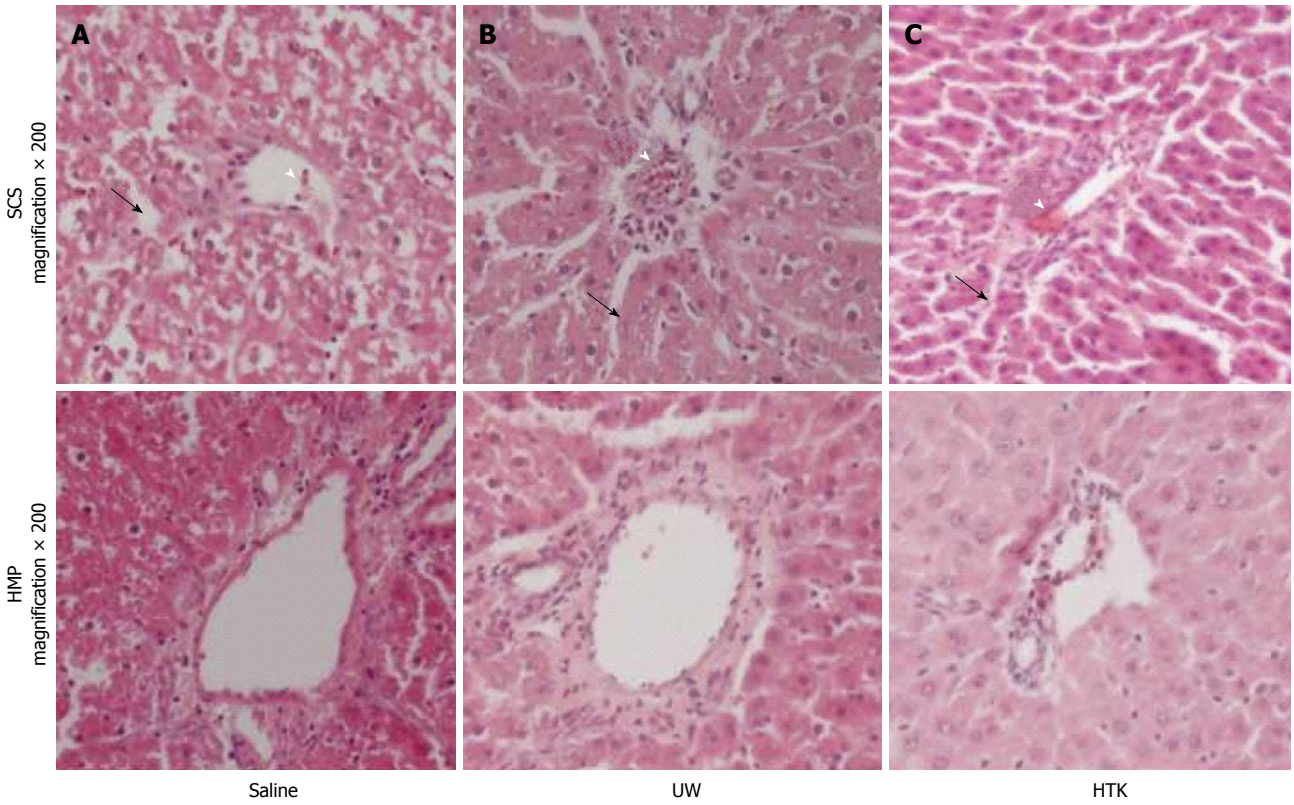

Figure 2 Histopathological appearance of the livers in the studies at 6 h after perfusion (hematoxylin and eosin staining, original magnification × 200). A: Saline; B: UW; C: HTK. Sinusoid lining (arrow) and congestion of the sinusoids and central vein (white arrow head).

by reactive oxygen species (ROS)[9]. Here MDA was measured using MDA kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) to evaluate the lipid peroxidation in liver tissue according to the manufacturer's protocols. All assays were performed with a Beckman Coulter DU-800 Spectrophotometer (USA). After 6 h of preservation, the left lateral lobe of the liver was weighed [wet weight (W), g] immediately after removal. The lobe was then dried overnight at 60 ℃ and reweighed [dry weight (D), g].

ATP levels and portal vein resistance (VR)

Liver tissue adenosine triphosphate (ATP) levels represent a good indicator of mitochondrial respiratory function and were measured using ATP kit (Beyotime, JiangSu, China) according to manufacturer's instructions. Portal VR (mmHg · min/mL) was calculated as portal vein pressure (mmHg)/velocity (mL/min).

Statistical analysis

All experimental results are shown as mean ± SD. Statistical differences were calculated using one-way analysis of variance (ANOVA) followed by the Least Squares Difference (LSD) or Dunnett's test for multiple comparisons. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Comparison of HMP and SCS

HMP improves the architecture and function of donor grafts compared to SCS regardless of the perfusate: Using saline as the perfusate, compared to SCS, HMP reduced the congestion around sinusoids and central vein, maintained sinusoid lining in morphology (Figure 2A) and decreased ALT, AST and LDH release, especially during the 3-6 h period (Figure 3). Similar results were found using UW (Figure 2B, Figure 4) and HTK (Figure 2C, Figure 5) as the perfusate.

HMP decreases MDA levels regardless of the perfusate: The MDA levels determined as an indirect measurement of oxidative damage induced by ROS in the HMP and SCS livers are shown in Figure 6. HMP induced significantly lower MDA level compared to SCS regardless of the perfusate (saline, 2.79 ± 0.30 *vs* 1.09 ± 0.09, $P = 0.008$; UW, 3.01 ± 0.77 *vs* 1.23 ± 0.68, $P = 0.005$; HTK, 3.30 ± 0.52 *vs* 1.56 ± 0.22, $P = 0.006$).

Comparison of HMP subgroups

HTK shows less portal VR than UW and saline: Portal VR among the HMP subgroups is shown in

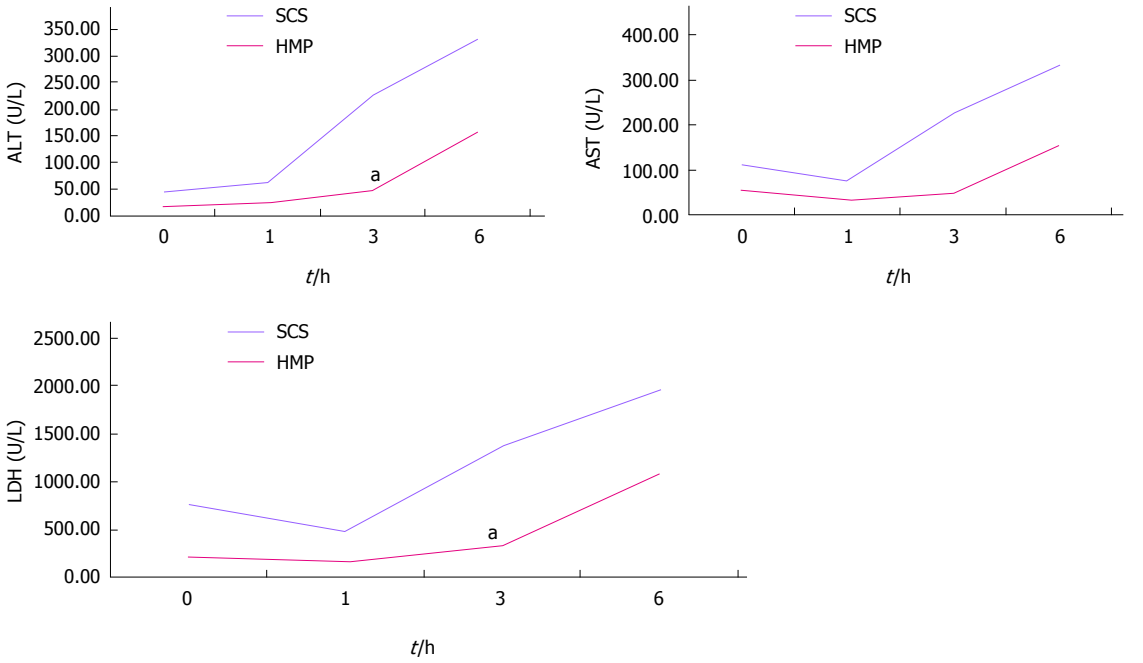

Figure 3 Levels of aspartate aminotransferase, alanine transaminase and lactate dehydrogenase during 6 h of preservation with saline perfusates (static cold storage *vs* hypothermic machine perfusion: Alanine transaminase 3, 225.00 ± 105.62 *vs* 49.50 ± 18.50, *P* = 0.047; LDH3, 1362.17 ± 563.30 *vs* 325.75 ± 147.43, *P* = 0.041). [a]Statistically significant difference. ALT3 means the ALT level at 3 h after liver preservation. AST: Aspartate aminotransferase; ALT: Alanine transaminase; LDH: Lactate dehydrogenase; SCS: Static cold storage; HMP: Hypothermic machine perfusion.

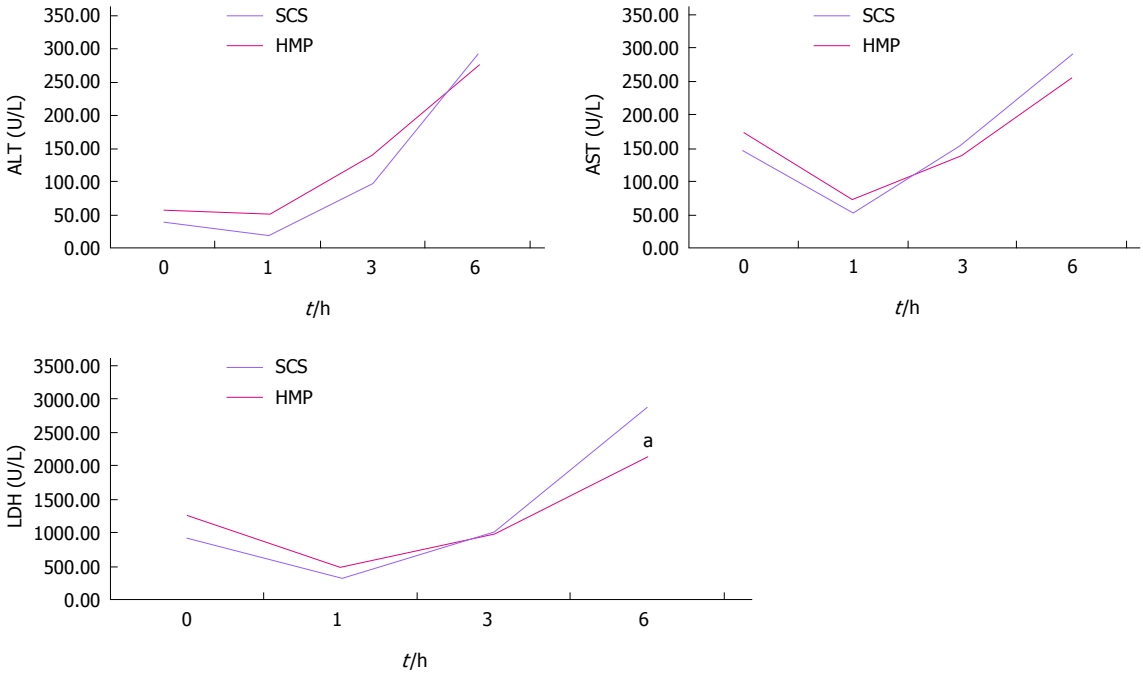

Figure 4 Levels of aspartate aminotransferase, alanine transaminase and lactate dehydrogenase during 6 h of preservation with University of Wisconsin cold-storage solution perfusates (static cold storage *vs* hypothermic machine perfusion: Lactate dehydrogenase 6, 2880.14 ± 948.46 *vs* 2135.00 ± 174.27, *P* = 0.049). [a]Statistically significant difference. LDH6 means the LDH level at 6 h after liver preservation. AST: Aspartate aminotransferase; ALT: Alanine transaminase; LDH: Lactate dehydrogenase; SCS: Static cold storage; HMP: Hypothermic machine perfusion.

Figure 7. The VR level gradually decreased among the saline, UW and HTK groups, with HTK showing the lowest VR (*vs* saline, 3.41 ± 0.49 *vs* 5.00 ± 0.38, *P* < 0.001; *vs* UW, 3.41 ± 0.49 *vs* 4.52 ± 0.63, *P* = 0.007)

UW reduces edema most efficiently, while HTK maintains ATP levels best: The D/W ratios and ATP levels among the HMP subgroups are shown in Figure 8. The D/W ratio gradually decreased between

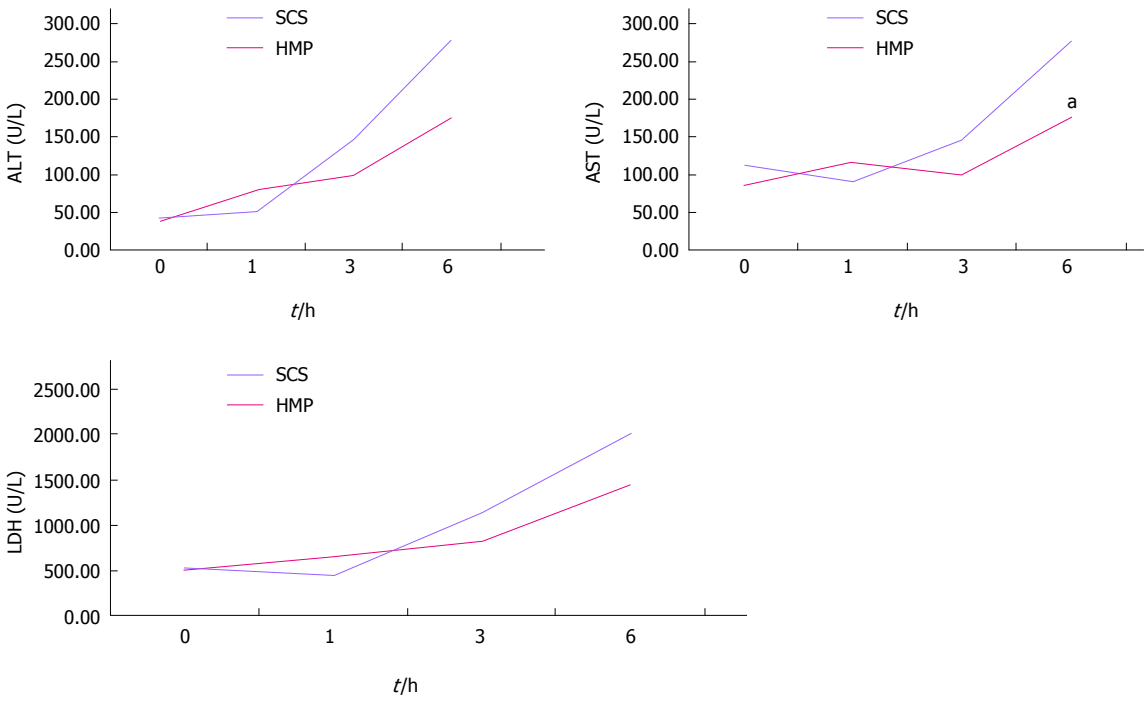

Figure 5 Levels of aspartate aminotransferase, alanine transaminase and lactate dehydrogenase during 6 h of preservation with histidine-tryptophan-ketoglutarate solution perfusates (static cold storage *vs* hypothermic machine perfusion: Aspartate aminotransferase 6, 307.50 ± 52.95 *vs* 185.20 ± 20.46, *P* = 0.041). [a]Statistically significant difference. AST6 means the AST level at 6 h after liver preservation. AST: Aspartate aminotransferase; ALT: Alanine transaminase; LDH: Lactate dehydrogenase; SCS: Static cold storage; HMP: Hypothermic machine perfusion.

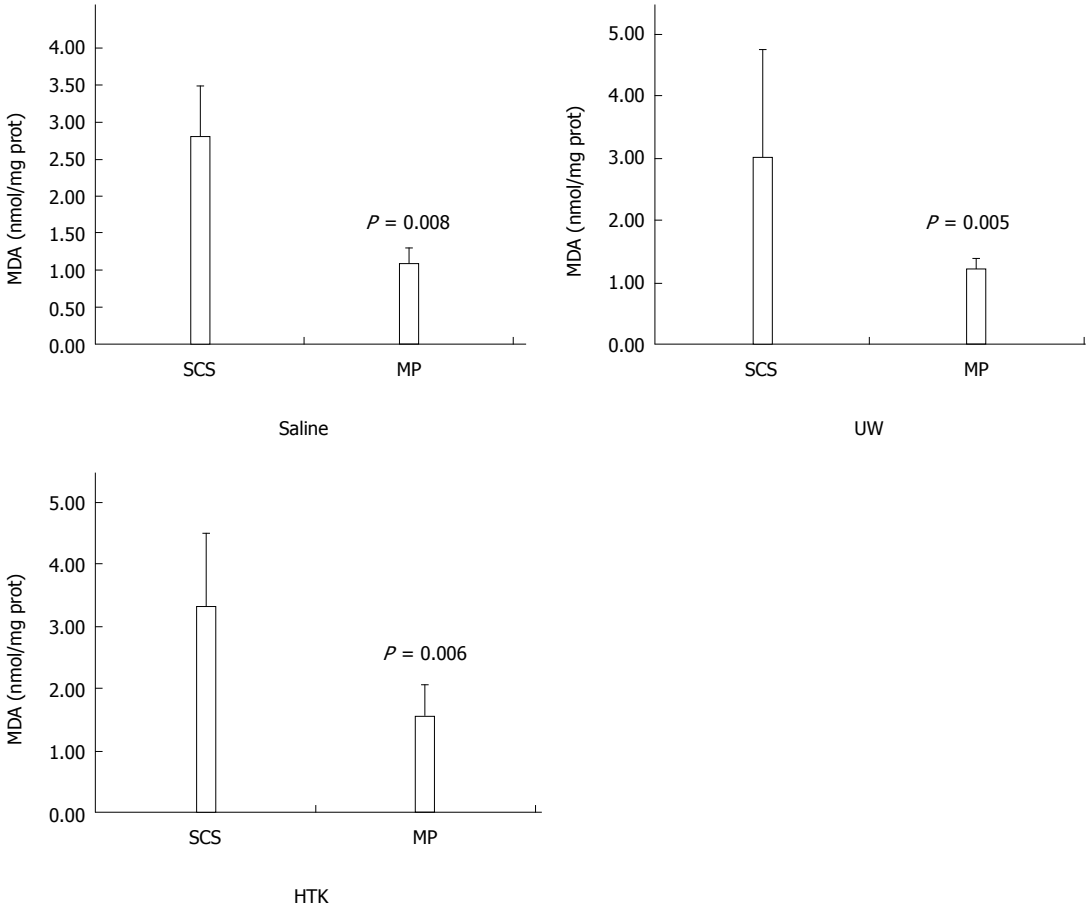

Figure 6 Levels of malondialdehyde in all groups at 6 h after perfusion. UW: University of Wisconsin cold-storage solution; HTK: Histidine-tryptophan-ketoglutarate solution.

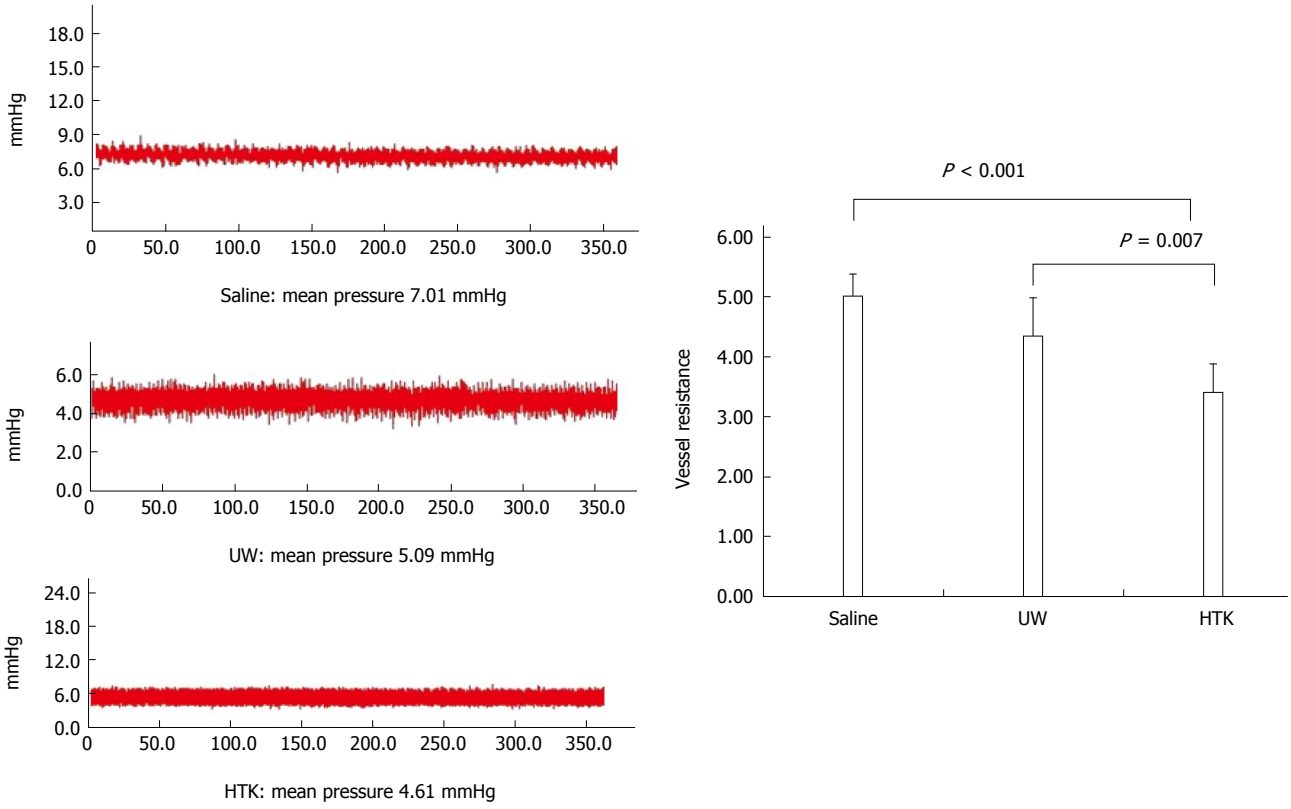

Figure 7 Portal vein resistance among hypothermic machine perfusion subgroups. The left panel shows a classical pressure record during perfusion from each subgroup while the right panel shows the results of statistical analysis. UW: University of Wisconsin cold-storage solution; HTK: Histidine-tryptophan-ketoglutarate solution.

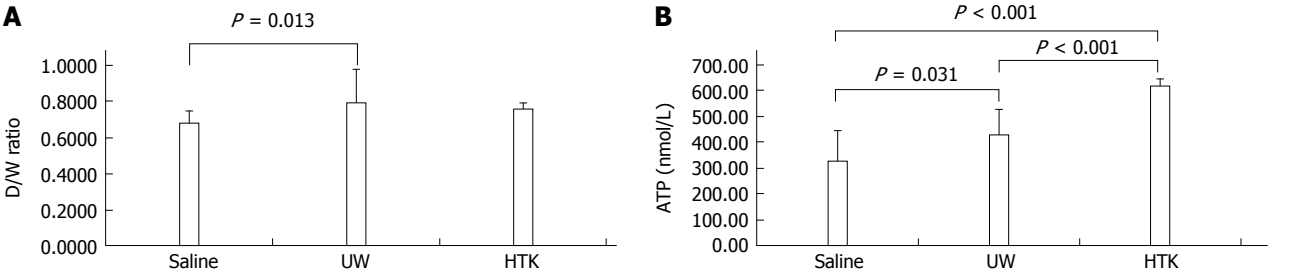

Figure 8 Dry/wet weight ratio (A) and adenosine-triphosphate levels (B) among hypothermic machine perfusion subgroups. D/W: Dry/wet weight ratio; UW: University of Wisconsin cold-storage solution; HTK: Histidine-tryptophan-ketoglutarate solution.

the subgroups perfused with UW, HTK and saline, indicating that UW was associated with the least edema, followed by HTK and then saline, which was associated with the most edema (Figure 8A, UW *vs* saline, 0.68 ± 0.02 *vs* 0.79 ± 0.05, $P = 0.013$). The ATP levels gradually increased between the subgroups perfused with saline, UW and HTK, demonstrating that HTK and UW maintained ATP level more effectively than saline, with HTK providing the best effect (Figure 8B, saline *vs* HTK, 327.43 ± 44.66 *vs* 622.60 ± 29.11, $P < 0.001$; saline *vs* UW, 327.43 ± 44.66 *vs* 301.80 ± 37.68, $P = 0.031$; UW *vs* HTK, 301.80 ± 37.68 *vs* 622.60 ± 29.11, $P < 0.001$)

DISCUSSION

Currently, more than 5500 cases of LT are performed each year in Europe and in the United States[10,11] with 5-year patient survival rates exceeding 70% in most centers. The success of LT has subsequently resulted in a substantial worldwide organ shortage. To reduce the gap between the patients' need and the availability of donors, the use of "extended criteria donor" (ECD) grafts, which were previously considered unsuitable for transplantation, has regained interest. Consequently, there is a critical need for improved methods of organ preservation. Currently, SCS and HMP are the

two major methods used for liver preservation. SCS is widely accepted because of its simplicity and for economic reasons; however, recent *ex vivo* and *in vivo* transplantation studies suggest that HMP provides improved preservation compared to SCS, especially for ECD grafts[12,13]. Nevertheless, the optimal conditions for HMP remain controversial due to a lack of consensus regarding the conditions in terms of perfusates, oxygenation, perfusion route (dual *vs* portal), and reperfusion pressure. Optimizing techniques would help to reduce preservation injury and increase the donor pool.

In this study, we established a stable, simplified HMP system and compared the performance of three perfusate solutions using this system. Two of the perfusates (UW and HTK) are commonly used in the clinical LT setting. Our results showed that HMP is superior to SCS in terms of AST, ALT, LDH release and MDA levels, regardless of the perfusate used, which is in accordance with previous reports[14,15]. Among the three perfusates evaluated, UW and HTK are superior to saline in HMP in terms of D/W ratio, VR and ATP levels. However, UW and HTK are associated with their own individual advantages and disadvantages. For instance, UW reduced edema more efficiently, but was associated with higher VR and ATP consumption than HTK, which may be due to its high viscosity and the hydroxyethyl starch (HES) component. Although UW is the most commonly used perfusate, its high viscosity may result in initial poor perfusion and incomplete distribution of UW between the intravascular space and the liver parenchyma in the donor grafts[16]. Furthermore, HES in UW causes hyperaggregating effects on erythrocytes, which hampers a complete washout from the liver and loss of ATP[17]. Compared with UW, HTK solution contains lower sodium and potassium concentrations, no HES and a much lower viscosity (approximately one-third that of UW), thus facilitating its release into the circulation[18]. Experimentally, studies have recently reported HTK is as good as or even better than UW for short periods of perfusion[19,20]. The low viscosity and absence of HES in HTK partially contribute to the relatively low VR and ATP consumption associated with its use as a perfusate[21].

Historically, a number of perfusates have been developed such as UW, HTK, Celsior, Cardiosol, or Custodiol[22]. Belzer *et al*[23], who proposed the concept of HMP, developed the first perfusate, UW, which remains the gold standard today. However, the intrinsic shortage of intracellular electrolytes and the HES component imposes marked changes in the ionic environment and produces high viscosity[2], resulting in deterioration of cellular components, with loss of ATP energy stores and alteration in the biochemical function and cellular architecture, which is even more deleterious in ECD[17]. Subsequently, other perfusates such as modified UW alone[24] and later with diluted, heparinized and oxygenated blood[25,26], Polysol[27], HTK or Custodiol-N solution[24] sometimes enriched with Kidney Perfusion Solution-1 and Aqix RS-I[28], were introduced to achieve better preservation. However, these perfusates have not resulted in significant improvements in the preservation of donor organs, with the exception of diluted blood. This perfusate has been shown to be safe and effective as an oxygen carrier in an animal model[29] and the first clinical HMP trial by Oxford University has shown an excellent outcome.

In this study, particular attention has been paid to the energy balance of the liver in its preservation. Recovered levels of ATP appear to be a good indicator of mitochondrial respiratory function. Improvement of mitochondrial condition, reflected here by augmentation of tissue ATP content during HMP, may result in improved tolerance to I/R injury[30]. Moreover, many clinical studies have shown that the preimplantation ATP content correlates with liver failure[31] and post-transplantation graft function[32].

There are some limitations of our study that should be noted. First, the time period for liver preservation (SCS or HMP) was only 6 h, while the benefits of UW associated with the ischemic period may extend to longer than 6 h. The selection of 6 h in our experimental design was mainly based on our clinical experience with 1500 LT cases, in which the optimized cold ischemia time was less than 6-8 h.

In conclusion, innovative and improved perfusates are an important approach to the future development of HMP techniques for LT. We established a stable HMP system for use in optimizing HMP conditions and demonstrated that HMP is superior to SCS in terms of both the architecture and function of the liver. Among the perfusates evaluated in our study, HTK was identified as the optimized machine perfusion solution (MPS). However, some optimized MPS as Polysol and diluted blood might have set a new standard for liver organ preservation solutions.

COMMENTS

Background

Although static cold-storage (SCS) is the gold standard for liver transplantation, hypothermic machine perfusion (HMP) is currently challenging the limitations of SCS. However, there is no consensus on the basic conditions used for HMP, including the optimal perfusates.

Research frontiers

Machine perfusion is an alternative preservation method for liver graft. Recent *ex vivo* and *in vivo* transplantation studies suggest that HMP provides improved preservation compared to SCS, especially for "extended criteria donor" grafts.

Innovations and breakthroughs

Plenty of articles compare UW and HTK in static cold storage, but we made first try to compare them in HMP setting and found HTK is superior to UW in terms of ATP maintaining and portal vein resistance.

Applications

Although countless studies show that HMP is superior to SCS in kidney

transplantation, SCS is still gold standard in liver transplantation. In the future stable HMP system will be taken into practice and the optimized perfusate will reach an agreement.

Terminology

HMP preserves the organ by a constant perfusion through its blood vessels with a machine perfusion solution.

Peer-review

The aim of this study was clear and relevant. It was relatively hard to evaluate the results because formation of UW and HTK was not explained. Comparison of UW and HTK would provide basis for the future experiments. Different reagents between UW and HTK would be a clue to the difference of storage performance. Investigation of the difference might pave the way to a better storage solution.

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ORIGINAL ARTICLE

Basic Study

Overexpression of pim-3 and protective role in lipopolysaccharide-stimulated hepatic stellate cells

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Author contributions: Zhang JX designed the research; Liu LH, Lai QN and Cheng B performed the research; Chen JY analyzed the data; Liu LH wrote the paper.

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Abstract

AIM: To investigate pim-3 expression in hepatic stellate cells (HSCs) stimulated by lipopolysaccharide (LPS), and its protective effect on HSCs.

METHODS: Rat HSC-T6 cells were stimulated by LPS. The effect of LPS on proliferation and apoptosis of HSC-T6 cells was investigated by methyl thiazoyl-tetrazolium (MTT) assay and flow cytometry after annexin V-fluorescein isothiocyanate/propidium iodide double staining. pim-3 mRNA and protein were detected by reverse transcriptase polymerase chain reaction and Western blotting at 48 h when HSC-T6 cells were stimulated with 1 μg/mL LPS for 0, 3, 6, 12, 24 and 48 h. The cells without stimulation served as controls. To study the effect of pim-3 kinase on HSC-T6 cells, si-pim3 (siRNA against pim-3) was transfected into HSC-T6 cells. HSC-T6 cells were subjected to different treatments, including LPS, si-pim3, or si-pim3 plus LPS, and control cells were untreated. Protein expression of pim-3 was detected at 48 h after treatment, and cell proliferation at 24 and 48 h by MTT assay. Apoptosis was detected by flow cytometry, and confirmed with caspase-3 activity assay.

RESULTS: LPS promoted HSC-T6 cell proliferation and protected against apoptosis. Significantly delayed upregulation of pim-3 expression induced by LPS

occurred at 24 and 48 h for mRNA expression (pim-3/β-actin RNA, 24 or 48 h *vs* 0 h, 0.81 ± 0.20 or 0.78 ± 0.21 *vs* 0.42 ± 0.13, $P < 0.05$), and occurred at 12 h and peaked at 24 and 48 h for protein expression (pim-3/GAPDH protein, 12, or 24 or 48 h *vs* 0 h, 0.68 ± 0.12, 1.47 ± 0.25 or 1.51 ± 0.23 *vs* 0.34 ± 0.04, $P < 0.01$). pim-3 protein was ablated by si-pim3 and upregulated by LPS in HSC-T6 cells at 48 h after treatment (pim-3/GAPDH: si-pim3, si-pim3 plus LPS or LPS *vs* control, 0.11 ± 0.05, 0.12 ± 0.05 or 1.08 ± 0.02 *vs* 0.39 ± 0.03, $P < 0.01$). Ablation of pim-3 by si-pim3 in HSC-T6 cells partly abolished proliferation (OD at 24 h, si-pim3 group or si-pim3 plus LPS *vs* control, 0.2987 ± 0.050 or 0.4063 ± 0.051 *vs* 0.5267 ± 0.030, $P < 0.01$; at 48 h 0.4634 ± 0.056 or 0.5433 ± 0.031 *vs* 0.8435 ± 0.028, $P < 0.01$; si-pim3 group *vs* si-pim3 plus LPS, $P < 0.01$ at 24 h and $P < 0.05$ at 48 h), and overexpression of pim-3 in the LPS group increased cell proliferation (OD: LPS *vs* control, at 24 h, 0.7435 ± 0.028 *vs* 0.5267 ± 0.030, $P < 0.01$; at 48 h, 1.2136 ± 0.048 *vs* 0.8435 ± 0.028, $P < 0.01$). Ablation of pim3 with si-pim3 in HSC-T6 cells aggravated apoptosis (si-pim3 or si-pim3 plus LPS *vs* control, 42.3% ±1.1% or 40.6% ± 1.3% *vs* 16.8% ± 3.3%, $P < 0.01$; si-pim3 *vs* si-pim3 plus LPS, $P > 0.05$), and overexpression of pim-3 in the LPS group attenuated apoptosis (LPS *vs* control, 7.32% ± 2.1% *vs* 16.8% ± 3.3%, $P < 0.05$). These results were confirmed by caspase-3 activity assay.

CONCLUSION: Overexpression of pim-3 plays a protective role in LPS-stimulated HSC-T6 cells.

Key words: Pim-3; Lipopolysaccharide; Hepatic stellate cell; Si-pim3

Core tip: Hepatic stellate cell (HSC)-T6 cells stimulated by lipopolysaccharide (LPS) showed overexpression of pim-3 kinase. Overexpression of pim-3 in LPS-stimulated HSC-T6 cells protected against apoptosis and promoted proliferation. Knockdown of *pim3* gene abolished proliferation of HSC-T6 cells and led to apoptosis. Overexpression of pim-3 induced by LPS play a protective role in rat hepatic stellate cells.

Liu LH, Lai QN, Chen JY, Zhang JX, Cheng B. Overexpression of pim-3 and protective role in lipopolysaccharide-stimulated hepatic stellate cells. *World J Gastroenterol* 2015; 21(29): 8858-8867 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i29/8858.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i29.8858

INTRODUCTION

Fibrosis and cirrhosis of the liver cause serious morbidity and mortality worldwide. Nearly all patients with chronic liver diseases experience liver fibrosis and some develop cirrhosis. Hepatic stellate cells (HSCs) are of pathogenetic relevance during the development, progression and regression of hepatic fibrosis. When the liver is injured, quiescent HSCs in the normal liver are activated. Activated HSCs secrete extracellular cell matrix (ECM) and inhibit ECM decomposition to promote progression of hepatic fibrosis. Promotion of apoptosis of activated HSCs may be an effective way to reverse fibrosis[1,2].

Lipopolysaccharide (LPS), which is found on the outer membrane of Gram-negative bacteria, is increased in the portal vein as the severity of hepatic fibrosis increases[3], due to increased portal vein pressure and gut permeability. LPS stimulates activity of HSCs and activates interaction of HSCs with Kupffer and endothelial cells to promote liver fibrosis and adjust portal vein pressure[4-6]. The cellular activation induced by LPS is accompanied with altered expression of several genes. Previous studies have revealed that LPS upregulates the activity of nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK) in activated HSCs[7-11], however, pim-3 kinase expression and its role in LPS-stimulated HSCs has not been reported.

pim kinase belongs to a serine/threoine protein kinase family that consists of pim-1, pim-2 and pim-3 and has been implicated in cell proliferation and apoptosis[12]. pim-3 kinase is overexpressed in both solid cancer cells and hematological malignancies, which contributes to tumor development through its anti-apoptosis and pro-proliferation functions[12]. In several normal cells, pim-3 kinase is upregulated by stress such as anoxia/reoxygenation injury, ischemia/reperfusion injury, or LPS, and protects against tissue injury[13,14]. Here, we investigated pim-3 expression and its protective role in LPS-stimulated HSCs.

MATERIALS AND METHODS

Chemicals

HSC-T6 is an immortal rat cell line transfected with SV40 T antigen vector containing sarcoma virus promoter[15]. The cell line was a generous gift from Scott L. Friedman. LPS (Sigma, St Louis, MO, United States) was used to stimulate HSC-T6 cells. siRNA (Biomics Biotechnologies, Shanghai, China) was used to study pim-3 function in HSC-T6 cells. Total protein extraction kits were purchased from Solarbio (Beijing, China). The primary antibody to pim-3 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, United States). Caspase-3 Activity Assay kit was from Beyotime Institute of Biotechnology (Nantong, China).

Cell culture

HSC-T6 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) at 37 ℃ in a humidified 5% CO_2

atmosphere. The cultures were passaged after 80% confluence every 3 d.

Methyl thiazoyltetrazolium assay

The determination of cell proliferation was based on methyl thiazoyltetrazolium (MTT) metabolism. HSC-T6 cells were seeded into 96-well plates at 10^4 cells/well with 0.75% FBS for 24 h, as described previously, with some modification[16]. At the designated time, 20 μL MTT (5 mg/mL) was added to each well and the medium was removed after 4 h following addition of 150 μL DMSO to dissolve the dye for 10 min. The absorbance of each well was read at 490 nm by a spectrophotometer (Thermo Fisher Scientific, Shanghai, China). The experiments were performed in triplicate.

Detection of apoptosis

Apoptosis was measured using AnnexinV-fluorescein isothiocyanate (FITC)/PI apoptosis detection kit I (BD, San Jose, CA, United States). After 48 h treatment with different stimuli, the cells were digested by trypsin without EDTA and collected by centrifugation at 300 × *g* for 5 min. Cells were washed twice with cooled PBS and resuspended with 100 μL binding buffer per 10^5 cells. Following incubation with 5 μL Annexin V-FITC and 5 μL PI solution in a dark room at room temperature for 15 min, 400 μL binding buffer was added and shaken slightly. The samples were collected and 10^4 cells were analyzed by a FACSCalibur flow cytometer (BD).

Caspase-3 assay

Protein was extracted from the treated HSC-T6 cells and the concentration was determined by BCA protein assay kits (Thermo Fisher Scientific). Caspase-3 activity was measured using the Caspase-3 Activity Assay kit (Beyotime Institute of Biotechnology). Cell extracts were mixed with Ac-DEVD-pNA substrate for 2 h at 37 ℃ in 96-well plates prior to colorimetric measurement of p-nitroanilide product at 405 nm.

Semi-quantitative reverse transcriptase polymerase chain reaction

HSC-T6 cells were seeded in six-well plates. After culturing for 12 h, cells received different treatments following serum starvation with 0.75% FBS. After treatment, total RNA was extracted from the cells using an RNA simple Total RNA kit (Tiangen, Beijing, China). The first strand of cDNA was synthesized with the reverse-transcript kit (Takara, Dalian, China). The following primers were used: (1)pim-3: forward: 5′-CACTGACTTTGATGGCACCC-3′ reverse: 5′-ATGCCCAGACGAAGACCA-3′(product of 770bp) (2) β-actin: forward: TCAGGTCATCACTATCGGCAAT reverse: AAAGAAAGGGTGTAAAACGCA (product of 432 bp). PCR was performed in a 25-μL reaction mixture containing 1 μL cDNA, 0.5 μL each primer, 0.25 μL rTaq DNA polymerase, and 2.0 μL dNTP. The PCR was performed with the following thermal cycling conditions: (1) denaturation at 95 ℃ for 5 min; (2) 35 cycles of denaturation at 94 ℃ for 45 s; and (3) primer annealing at 55 ℃ for 45 s and primer extension at 72 ℃ for 60 s, with a final extension at 72 ℃ for 10 min. The PCR products were electrophoresed in a 1.5% agarose gel containing ethidium bromide and visualized with UV light. The bands in the gels were quantified with Quantity one 4.62 and the level of a particular cDNA was normalized to that of β-actin product.

Protein expression determination

HSC-T6 cells were seeded in six-well plates. After culturing for 12 h, cells received different treatments following serum starvation with 0.75% FBS. After treatment, the cell pellets were collected, washed three times with ice-cold PBS, and resuspended in lysis buffer to extract protein. Protein concentration was determined by BCA protein assay kits (Thermo Fisher Scientific). The protein solution was heat-denatured with an equal volume of 2 × SDS loading buffer for 5 min and separated on 12% SDS-PAGE. The protein was then electro-transferred onto PVDF membranes. After blocking with 5% skimmed milk in PBS at 4 ℃ overnight, the membrane was incubated with each primary antibody, followed by incubation with a horseradish-peroxidase-conjugated secondary antibody. The membrane was then exposed to X-ray film and the quantification of the bands was carried out by Quantity one 4.62. GAPDH was used as an internal control for loading.

Assessment of LPS effect on HSC-T6 cells

HSC-T6 cells were subjected to LPS (*Escherichia coli* 055:B5) treatment at different concentrations (10 ng/mL, 100 ng/mL, 1 μg/mL or 5 μg/mL) for 24 or 48 h following starvation with 0.75% FBS. MTT assay was conducted to achieve the optimum concentration of LPS for promotion of HSC-T6 cell proliferation. Apoptosis was detected by flow cytometry at 48 h after treatment with 1 μg/mL LPS. Reverse transcriptase polymerase chain reaction (RT-PCR) and western blotting were performed to detect pim-3 expression at 48 h when HSC-T6 cells were stimulated with 1 μg/mL LPS by different time-course (0 , 3 , 6 , 12 , 24 and 48 h). The cells without stimulation served as controls.

RNA interference protocol

Short interfering RNA (siRNA) was synthesized by Biomics Biotechnologies. siRNA duplexes were designed to target AA(19)UU sequences in the open reading frame of mRNA encoding pim-3. Three siRNA against pim-3 (si-pim3) and one scrambled siRNA were transiently transfected into HSC-T6 cells with Lipofectamine 2000 transfection regent (Invitrogen, Carlsbad, CA, United States). One day before transfection, HSC-T6 cells were cultured in DMEM with no antibiotics, then in medium with serum-free complexes containing siRNA and Lipofectamine

Table 1 Effect of lipopolysaccharide on hepatic stellate cells-T6 proliferation assessed by methyl thiazoyltetrazolium assay

Parameter	24 h (OD)	48 h (OD)
Control	0.5033 ± 0.023	0.8853 ± 0.021
LPS (10 ng/mL)	0.5998 ± 0.019[a,b]	0.9813 ± 0.037[a,b]
LPS (100 ng/mL)	0.6002 ± 0.038[a,b]	1.1126 ± 0.050[a,b]
LPS (1000 ng/mL)	0.7032 ± 0.066[b]	1.2469 ± 0.0525[b]
LPS (5000 ng/mL)	0.6625 ± 0.015[b]	1.2141 ± 0.018[b]

Data are expressed as mean ± SD (n = 6). [a]P < 0.05 *vs* LPS, 1000 ng/mL, [b]P < 0.01 *vs* control.

2000 (20 pmol siRNA to 1 μL Lipofectamine 2000) for 6 h, followed by DMEM with 10% FBS. Forty-eight hours later, the cells were harvested and lysed and pim-3 mRNA and protein expression was detected by RT-PCR and western blotting to select the perfect siRNA duplex. The selected siRNA duplex (sense chain: 5'-UUCUCCGAACGUGUCACGUdTdT-3' antisense chain 5'-ACGUGACACGUUCGGAGAAdTdT-3') and the scrambled siRNA duplex (sense chain: 5'-UUCUCCGAACGUGUCACGUdTdT-3', antisense chain: 5'-ACGUGACACGUUCGGAGAAdTdT-3') was further blasted to search against another rat genome sequence to ensure its targets specificity. Experiments were divided into the following groups. Control group: HSC-T6 cells incubated without treatment. Liposome group: HSC-T6 cells incubated with equivalent liposome. Scramble group: scrambled RNA transfected into HSC-T6 cells. si-pim3 group: si-pim3 was transfected into HSC-T6 cells. LPS group: HSC-T6 cells treated with 1 μg/mL LPS. si-pim3 plus LPS group: si-pim3 was transfected into HSC-T6 cells, then treated with 1 μg/mL LPS. HSC-T6 cells were harvested at the designated time and cell proliferation (at 24 or 48 h after treatment), protein expression (at 48 h), and apoptosis (at 48 h) were determined. Each experiment was repeated three times.

Statistical analysis

Values were expressed at mean ± SD from duplicate samples. The difference in the means between two groups was tested by the Students' t test (two tailed), and that between the groups (above three groups) was tested by one-way ANOVA followed by Student-Newman-Keul test; P < 0.05 was considered statistically significant.

RESULTS

Effect of LPS on proliferation and apoptosis of HSC-T6 cells

Proliferation of HSC-T6 cells treated with LPS at 10 ng/mL, 100 ng/mL, 1 μg/mL and 5 μg/mL was 1.19-, 1.19-, 1.39- and 1.31-fold that of the control group at 24 h after treatment; and 1.10-, 1.25-, 1.41- and 1.37-fold that at 48 h after treatment (Table 1). Proliferation with 1 μg/mL LPS was the highest. Our results show that LPS promotes HSC-T6 cell proliferation and the optimum concentration is 1 μg/mL.

Apoptosis was detected by flow cytometry following Annexin-FITC/PI double staining, and $FITC^+PI^-$ spots represented early apoptosis, while $FITC^+PI^+$ spots represented late apoptosis. Total apoptosis rate of HSC-T6 cells treated with LPS was lower than that of the control group (control *vs* LPS, 16.3% ± 2.4% *vs* 8.3% ± 2.3%, P < 0.05), which suggests that LPS has an inhibitory effect on HSC-T6 apoptosis (Figure 1).

Overexpression of pim-3 induced by LPS in HSC-T6 cells

Expression of pim-3 mRNA in LPS-stimulated HSC-T6 cells at 3, 6, 12, 24 and 48 h after treatment was 0.95-, 1.28-, 1.63-, 1.94- and 1.84-fold that of the control group, respectively (Figure 2). Expression was significantly increased at 24 and 48 h after treatment (P < 0.05, compared to that at 0 and 3 h). Expression of pim-3 protein in LPS-treated cells at 3, 6, 12, 24 and 48 h after treatment was 1.67-, 1.42-, 2.01-, 4.30- and 4.42-fold that of the control group, respectively. Expression increased significantly at 12 h (P < 0.05, compared with 0 h), reached a plateau at 24 h, and was sustained at a high level at 48 h (P < 0.01, compared with 0 h). The results clearly demonstrated that LPS significantly increases expression of pim-3 in HSC-T6 cells at transcriptional and translational levels and delays overexpression of pim-3.

Expression of pim-3 in RNA interference protocol

In order to clarify the role of endogenous pim-3 in HSC-T6 cells, we tested cell proliferation and apoptosis under the condition of pim-3 ablation by RNA interference (RNAi) and overexpression of pim-3 induced by LPS. Three si-pim3 duplexes were used and pim-3 mRNA and protein levels were measured in HSC-T6 cells at 48 h after si-pim3 transfection. si-pim3 was selected depending on the experiment for its strongest inhibition. The selected si-pim3 duplex (sense chain: 5'-UUCUCCGAACGUGUCACGUdTdT-3' antisense chain 5'-ACGUGACACGUUCGGAGAAdTdT-3') could reduce about 70% expression of pim-3 protein. pim-3 expression was confirmed by western blotting, and pim-3 protein was ablated by si-pim3 and upregulated by LPS at 48 h after treatment (Figure 3) (pim-3/GAPDH: si-pim3 or si-pim3 plus LPS or LPS *vs* control, 0.11 ± 0.05 or 0.12 ± 0.05 or 1.08 ± 0.02 *vs* 0.39 ± 0.03, P < 0.01).

Effect of pim-3 kinase on HSC-T6 cell proliferation

To determine the effect of pim-3 kinase on HSC-T6 cell proliferation, we compared proliferation among different groups treated with si-pim3 transfection (ablation of pim-3), LPS (overexpression of pim-3), or LPS combined si-pim3 transfection (ablation of pim-3).

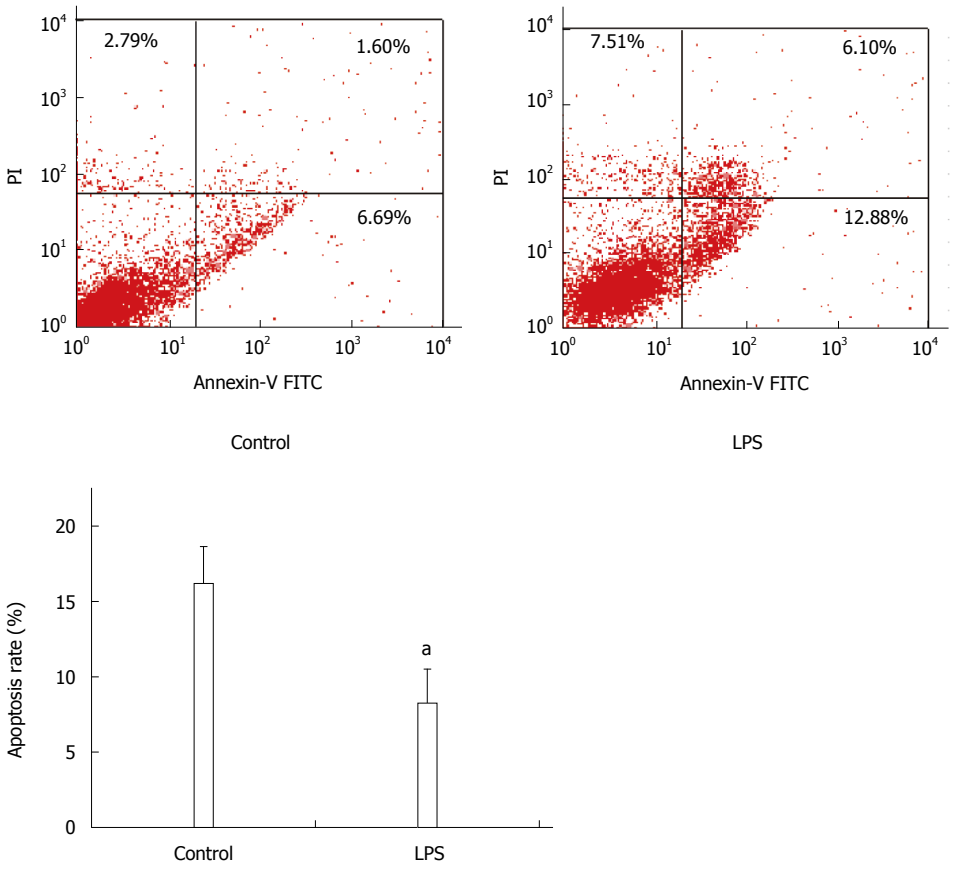


Figure 1 Protective effect of lipopolysaccharide on apoptosis of hepatic stellate cell-T6 cells. Cells were treated with 1 μg/mL LPS for 48 h. Upper panel, flow cytometry according to annexin-fluorescein isothiocyanate/PI double staining; lower panel, apoptosis rate. Data are expressed as mean ± SD. $n = 3$, $^{a}P < 0.05$ ($P = 0.013$) *vs* control. LPS: lipopolysaccharide.

Table 2 Effect of ablation of pim-3 on HSC-T6 cell proliferation assessed by methyl thiazoyltetrazolium assay

Group	24 h (OD)	48 h (OD)
Control	0.5267 ± 0.030	0.8435 ± 0.028
Liposomes	0.5749 ± 0.028	0.8552 ± 0.014
si-pim3	0.2987 ± 0.050^{b}	0.4634 ± 0.056^{b}
scrambled	0.5062 ± 0.066	0.8069 ± 0.039
si-pim3 plus LPS	0.4063 ± 0.051^{a,b}	0.5434 ± 0.031^{b}
LPS	0.7435 ± 0.028^{b}	1.2136 ± 0.048^{b,c}

Data are expressed as mean ± SD ($n = 6$). $^{a}P < 0.05$ *vs* si-pim3 group; $^{b}P < 0.01$ *vs* control group; $^{c}P < 0.01$ LPS *vs* other groups.

HSC-T6 cell proliferation in the si-pim3 group and si-pim3 plus LPS group was significantly decreased, compared with the control group (OD: si-pim3 or si-pim3 plus LPS *vs* control, at 24 h, 0.2987 ± 0.050 or 0.4063 ± 0.051 *vs* 0.5267 ± 0.030, $P < 0.05$; at 48 h, 0.4634 ± 0.056 or 0.5434 ± 0.031 *vs* 0.8435 ± 0.028, $P < 0.05$), whereas proliferation in the LPS group was significantly increased, compared with the control group (OD: LPS *vs* control, at 24 h, 0.7435 ± 0.028 *vs* 0.5267 ± 0.030, $P < 0.01$; at 48 h, 1.2136 ± 0.048 *vs* 0.8435 ± 0.028, $P < 0.01$) (Table 2). These results indicated that ablation of pim-3 in the si-pim3 group inhibited HSC-T6 cell proliferation, and overexpression of pim-3 in the LPS group promoted HSC-T6 proliferation, which suggests that endogenous pim-3 has potential pro-proliferative activity in HSC-T6 cells. HSC-T6 cell proliferation in the si-pim3 plus LPS group was significantly increased, compared with the si-pim3 group ($P < 0.05$), however, they had similar expression of pim-3, which means that LPS has another approach to promote cell proliferation independent of pim-3 kinase.

Effect of pim-3 kinase on HSC-T6 cell apoptosis

The apoptosis rate of HSC-T6 cells in the LPS group with overexpression of pim-3 was significantly lower than that of the control group (LPS *vs* control, 7.32% ± 2.1% *vs* 16.8% ± 3.3%, $P < 0.05$) (Figure 4). In contrast, the apoptosis rate of the si-pim3 group with ablation of pim-3 was remarkably higher than that of the control group (si-pim3 *vs* control, 42.3% ± 1.1% *vs* 16.8% ± 3.3%, $P < 0.01$), but similar to that of the si-pim3 plus LPS group (si-pim3 *vs* si-pim3 plus LPS, 42.3% ± 1.1% *vs* 40.6% ± 1.3%, $P > 0.05$). These results demonstrated that ablation of pim-3 can aggregate HSC-T6 cell apoptosis and endogenous pim-3 kinase may protect HSC-T6 cells from apoptosis.

Caspase-3 is a cell death executioner, which can be activated by various apoptosis signals. To confirm the

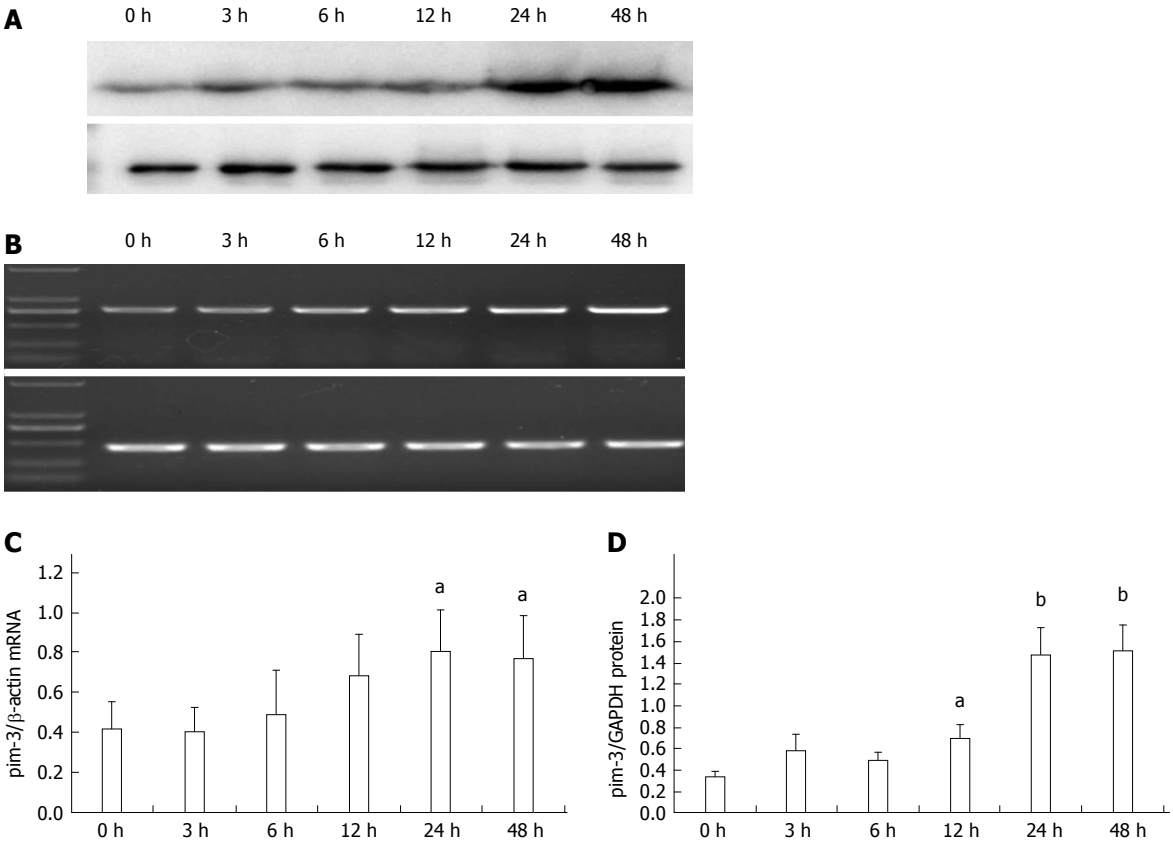
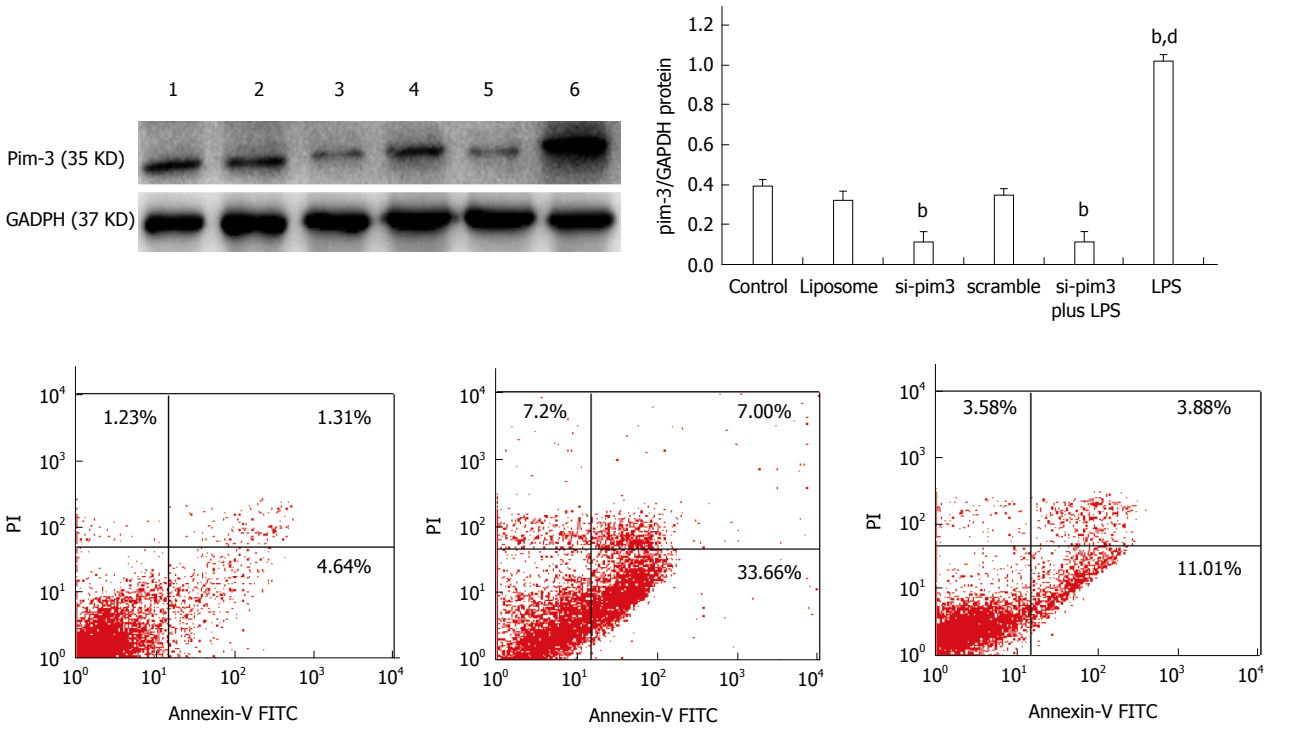


Figure 2 Overexpression of pim-3 in lipopolysaccharide-stimulated hepatic stellate cells-T6 cells. Cells were treated at different times, with 1 μg/mL lipopolysaccharide for 0, 3, 6, 12, 24 and 48 h, and samples were collected at the same final time of 48 h. mRNA and protein level of pim-3 expression was detected by RT-PCR and Western blotting, respectively. A: Representative mRNA expression; B: Representative protein expression; C: Relative level of pim-3 was normalized to value obtained for β-actin mRNA expression; D: Relative level of pim-3 was normalized to value obtained for GADPH protein expression. The data are expressed in line chart in order to reflect the expression change over time. Data are expressed as mean ± SD (n = 3). $^{a}P < 0.05$ *vs* 0 h; $^{b}P < 0.01$ *vs* 0 h.



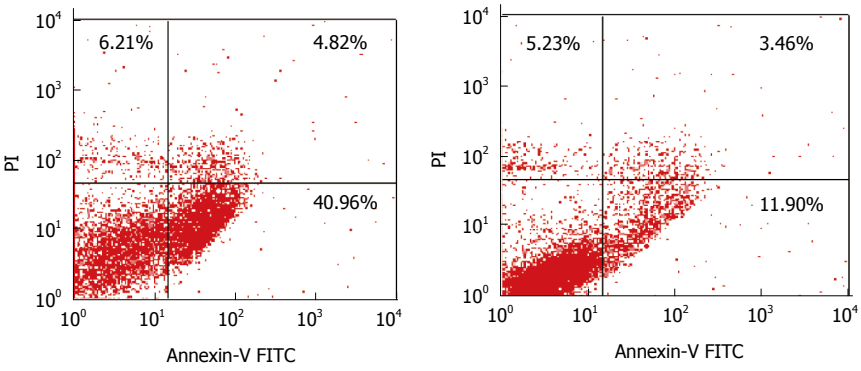


Figure 3 Effect of si-pim3 on pim-3 expression. Cells were treated at different times and pim-3 protein was detected at 48 h by western blotting. Lane 1: control; Lane 2: liposomes; Lane 3: si-pim3; Lane 4: scrambled; Lane 5: si-pim3 plus LPS; Lane 6: LPS. Upper panel, representative Western blotting results; lower panel, relative expression levels of proteins, normalized against GADPH. Data are expressed as mean ± SD (n = 3). $^{b}P < 0.01$ *vs* control, $^{d}P < 0.01$ LPS *vs* other groups.

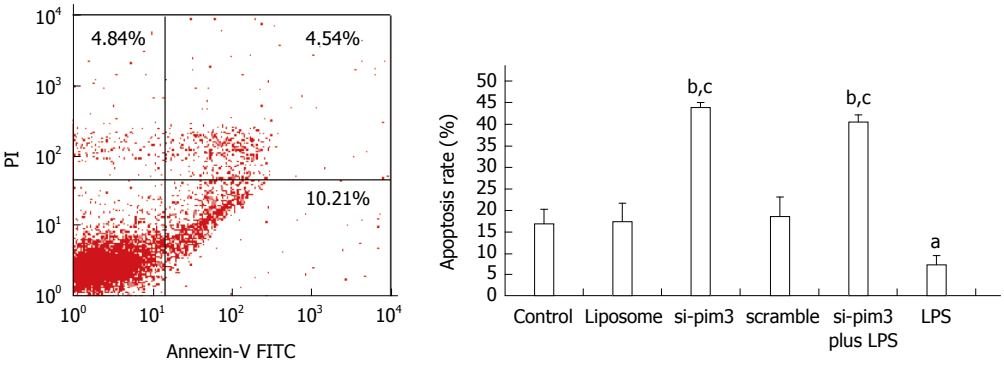


Figure 4 Effect of ablation of pim-3 on hepatic stellate cells-T6 cell apoptosis. Cells were treated with si-pim3, LPS or si-pim3 plus LPS for 48 h, and apoptosis was detected by flow cytometry followed by annexin V-FITC/PI double staining. A: Representative flow cytometry following Annexin V-FITC and PI staining; B: Data are expressed in a histogram in order to reflect the apoptosis change among the six groups. Data are expressed as mean ± SD. n = 3, $^{a}P < 0.05$ LPS *vs* control; $^{b}P < 0.01$ *versus* control; $^{c}P < 0.01$ *vs* LPS group.

Table 3 Effect of ablation of pim-3 on caspase-3 activity of hepatic stellate cells-T6 cells

Groups	Caspase-3 activity (U)
Control	5.12 ± 1.33
Liposomes	4.95 ± 1.62
si-pim3	13.24 ± 2.81^{b,d}
scrambled	5.28 ± 0.93
si-pim3 plus LPS	11.35 ± 0.85^{b,d}
LPS	3.21 ± 0.55^{a}

Data are expressed as mean ± SD (n = 6). $^{a}P < 0.05$ *vs* control group; $^{b}P < 0.01$ *vs* control group; $^{d}P < 0.01$ *vs* LPS.

results from flow cytometry following Annexin V-FITC/PI staining, we quantified the activity of caspase-3. Caspase-3 activity in the LPS group was lower than in the control group (Table 3). In contrast, the activity in the si-pim3 group was significantly higher than in the control group ($P < 0.05$), and similar to that in the si-pim3 plus LPS group. All the results were consistent with those from flow cytometry following Annexin-FITC/PI double staining.

DISCUSSION

To the best of our knowledge, the present work is the first report about the expression of pim-3, as well as its role in HSCs treated with LPS. In this study, HSC-T6 cells stimulated by LPS showed overexpression of pim-3 kinase. Overexpression of pim-3 in LPS-stimulated HSC-T6 cells protected against apoptosis and promoted proliferation, however, knockdown of pim3 gene by si-pim3 abolished proliferation of HSC-T6 cells and led to apoptosis. These results suggest that overexpression of pim-3 induced by LPS has a protective role in rat HSCs.

Endotoxin in the portal vein and circulating blood is increased by aggravation of chronic liver disease and hepatic fibrosis[3], and contributes to hepatic fibrosis[5,17]. LPS could indirectly activate HSCs and protect against apoptosis by soluble mediators from Kupffer cells[18-20], or apoptotic bodies from damaged hepatocytes[21,22]. Activated HSCs have several phenotypes, including proliferation, fibrogenesis, contractility, inflammatory signaling,

and chemotaxis[23]. LPS also directly activates HSCs, and the cells show the inflammatory phenotype and secrete cytokines and chemokines[7,24]. Here, we studied the effect of LPS on proliferation and apoptosis of HSCs. The proliferation of primary activated HSCs in response to LPS, assessed by [3H]-thymidine incorporation, is unchanged[7], while that from HSC lines assessed by MTT is increased[25]. The difference may be due to differently derived cells and detection assays. In our study, LPS has a beneficial effect on proliferation of HSC-T6 cells and protects cells from apoptosis.

Antibiotic use is a way to eliminate gut-derived endotoxin, and animal experiments have demonstrated its efficacy in hepatic fibrosis[5]. However, long-term use of antibiotics increases the possibility of drug resistance, and presently no antibiotics are approved for use against hepatic fibrosis. Thus, it may be necessary to explore alterations in survival gene expression in LPS-treated HSCs, to help find new ways to avoid the effect of LPS on hepatic fibrosis. Previous studies have demonstrated that LPS upregulates secretion of tissue inhibitor of metalloproteinase (TIMP-)1[9] (necessary for prevention of HSC apoptosis[26]) and interleukin-6 (necessary for HSC transdifferentiation[27]), and upregulates expression of intracellular survival signal molecules, such as NF-κB, extracellular signal-regulated kinase (ERK) and C-Jun N-terminal kinase[11,16].

pim kinase is one of the serine/threoine protein kinase family, which at least includes pim-1, pim-2 and pim-3. The three members are well conserved in vertebrates and show structural similarity and functional overlap. They all have a role in promoting cell growth and inhibiting apoptosis. Many tumor cells overexpress pim-3, such as solid cancers and hematological malignancies[12]. Normal cells induced by special stimuli can also upregulate expression of pim-3 kinase, such as cardiomyocytes with anoxia/reoxgenation injury[13] , endothelial cell with tumor necrosis factor-α[28], and intestinal mucosa with LPS[14]. Our study clearly demonstrated that pim-3 expression is upregulated in HSCs treated with LPS. Unlike other survival signal molecules such as NF-κB and MAPK, which are characteristic of rapid and transient upregulation in LPS-stimulated HSCs[11], pim-3 expression shows late and persistent upregulation, which suggests that overexpression of pim-3 depends on other upstream signaling molecules. pim-3 kinase is involved in accelerating the cell cycle and protecting against apoptosis. pim-3 kinase can phosphorylate $p27^{kipl}$, inducing 14-3-3 binding and proteasome-dependent degradation, thus relieving the inhibition of the cell cycle and promoting proliferation[29]. pim-3 also phosphorylates BCL-xL/BCL-2-associated death promoter (BAD), inducing 14-3-3 binding and degradation, leading to release of Bcl-XL and Bcl-2[30,31], thus promoting cell survival[32]. Meanwhile, pim-3 can phosphorylate signal transducer and activator of transcription (STAT)3[33], promote antiapoptotic protein synthesis of Bcl-XL and survivin, leading to cell survival. pim-3 kinase is constitutively active and does not require post-translational modifications for the induction of kinase activity. Ablation of pim-3 by RNAi was used to explore its function in HSCs treated with LPS. Our results show that endogenous pim-3 could play a protective role in LPS-stimulated HSC-T6 cells, according to the results from ablation of pim-3 by si-pim3 and overexpression of pim-3 induced by LPS. The apoptosis rate of si-pim3-treated cells was similar to that of cells treated by si-pim3 plus LPS, which means that the antiapoptotic effect of LPS is completely inhibited by si-pim3. However, proliferation of si-pim3-treated cells was lower than that of the cells treated by si-pim3 plus LPS, although they had similar expression of pim-3, which suggests that cell proliferation induced by LPS has another mechanism independent of pim-3. LPS upregulates pim3 kinase and stimulates several other survival kinases, such as NF-κB and ERK. The results suggest that pim-3 kinase is downstream of other survival genes that are dependent on LPS, which coincides with the timing of pim-3 expression. Further study is needed to ascertain the relationship of pim-3 and other survival kinases.

In conclusion, the present study provides evidence that LPS can upregulate pim-3 expression in activated HSCs, and pim-3 expression can promote cell proliferation and inhibit apoptosis. Thus, pim-3 kinase may be an antifibrotic candidate target and blocker of pim-3 kinase, and an effective way to reverse liver fibrosis.

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COMMENTS

Background

pim-3 kinase is implicated in cell proliferation and anti-apoptosis. Upregulation of pim-3 expression often occurs in cancer and normal cells upon stress. Hepatic stellate cells (HSCs) are often challenged with gut-derived endotoxin.

Research frontiers

pim-3 kinase often plays a protective role when cells, such as cardiomyocytes, are challenged with anoxia or ischemic injury, or intestinal mucosa with lipopolysaccharide (LPS). Promotion of HSC apoptosis may be an effective way to reverse fibrosis. However, there is no research about pim-3 expression in HSCs induced by LPS, and its role in activated HSCs when challenged with LPS.

Innovations and breakthroughs

This is the first report about the expression of pim-3 as well as its role in HSCs treated with LPS. We demonstrated that overexpression of pim-3 is induced by LPS and plays a protective role in rat HSCs.

Applications

This study provided us with a new candidate target to relieve hepatic fibrosis.

Peer-review

This study is meaningful and the findings that pim-3 might be involved in LPS-stimulated hepatic stellate cells are interesting.

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ORIGINAL ARTICLE

Case Control Study

Circulating levels of vitamin D and colorectal adenoma: A case-control study and a meta-analysis

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Abstract

AIM: To examine the association between circulating 25-hydroxyvitamin D [25(OH)D] levels and colorectal adenoma in a case-control study and a meta-analysis.

METHODS: We conducted a matched case-control study (112 cases and 112 matched controls) and combined 15 studies, including our study, in a meta-analysis. The study-specific odds ratios (ORs) and 95% confidence intervals (CIs) were pooled using a random-effects model. In total, 5454 colorectal adenomas and 6656 controls were included in the meta-analysis.

RESULTS: In a meta-analysis including 14 previous studies and our study, we observed a significant inverse association between circulating 25(OH)D levels and colorectal adenoma (OR = 0.68; 95%CI: 0.54-0.82) when comparing the highest category with the lowest category. Stratification by adenoma location (proximal or distal adenoma) showed similar estimates. When we stratified by study region, the ORs (95%CIs) were 0.70 (0.52-0.88) in the US and 0.66 (0.34-0.97) in Asia.

CONCLUSION: These data suggest an inverse association between circulating 25(OH)D levels and colorectal adenoma in both Western and Asian populations.

Key words: 25-hydroxyvitamin D levels; Colorectal adenoma; Cancer prevention

Core tip: Growing evidence from epidemiologic studies suggests a preventive effect of vitamin D against colorectal cancer. Colorectal adenoma is considered to be a precursor lesion of colorectal cancer. We conducted a case-control study in Korean adults and also calculated a summary estimate through a meta-analysis to examine the association between circulating 25-hydroxyvitamin D[25(OH)D] levels and colorectal adenoma. We found an inverse association between circulating 25(OH)D levels and colorectal adenoma, and this association was consistent for Asian populations.

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INTRODUCTION

Vitamin D is synthesized in human skin that is exposed to ultraviolet light and is also obtained through supplements and several food sources. Because the vitamin D from food does not adequately reflect the vitamin D status of an individual, circulating concentrations of 25-hydroxyvitamin D [25(OH)D] may be a suitable measurement of vitamin D status. However, 1,25(OH)2D may not be a good indicator of vitamin D because it is tightly regulated by various factors[1].

Growing evidence suggests that low vitamin D levels are prevalent worldwide[2,3], partly because of an insufficient supply of vitamin D from natural food sources[4], a shift to sedentary lifestyles, and a lack of outdoor activities. Garland *et al*[5] suggested that exposure to solar radiation could be a protective factor for colon cancer in an ecologic study, and several epidemiologic studies found a reduction in colorectal cancer with better vitamin D status[6,7]. 1,25-dihydroxyvitamin D [1,25(OH)$_2$D], the active form of vitamin D, exerts its inhibitory effects on tumors in the normal and neoplastic colonic epithelium by affecting cell growth regulation, cell cycle regulation, and apoptosis and by interacting with proto-oncogenes and tumor suppressor genes[8].

The incidence rate of colorectal cancer has increased in some Asian countries, which have undergone dramatic lifestyle changes. For example, the colorectal cancer incidence has steadily increased in Korea by 4.7% annually from 1999 to 2010[9]. Colorectal cancer often develops from a colorectal adenoma in a process known as the adenoma-carcinoma sequence.

A considerable proportion of the East Asian population has low vitamin D status. The prevalence of < 20 ng/mL vitamin D levels was 56.0% in Korean adults of the Korean National Health and Nutrition Examination Survey 2008[10], 69.2% in the middle-aged or elderly Chinese population in a cross-sectional study[11], and 35.3% in postmenopausal Japanese women[12]. Despite the increase in colorectal cancer incidence and the low vitamin D status in Asian populations, only a few Asian studies on vitamin D and colorectal adenoma have been conducted. One Japanese study found a lower prevalence of colorectal adenoma with high vitamin D status during the winter season[13], and another Japanese study observed an inverse, but nonlinear, association[14]. One Korean case-control study performed an analysis among 143 age- and gender-matched case and control pairs and found an inverse association[15]. Although we reported a potential inverse association between circulating vitamin D levels and colorectal adenoma in a previous meta-analysis[16], a limited number of Asian studies did not allow us to examine whether this association observed was applied to Asian populations.

To determine whether circulating vitamin D levels are inversely associated with colorectal adenoma, we analyzed the association between colorectal adenoma and 25(OH)D levels among a matched case-control study of Korean adults, and we conducted a meta-analysis of 15 studies, including 14 previous published studies and our study.

MATERIALS AND METHODS

Case control study

Study population: We conducted a case-control study among 45- to 71-year-old men and women who underwent colonoscopy at a university hospital in Daegu, city of Korea, from August 2011 to September 2012. The size, subtype and number of colorectal adenomas were determined through colonoscopy and pathological examination. Polyps were classified as adenomatous, hyperplastic, or other nonadenomatous. Only adenomatous polyps were included as cases. We included both first and recurrent adenomas (4.7%). To minimize the influence of fasting status or sex, we performed 1:1 matching by fasting status and sex. As a result, a total of 112 cases and 112 matched-controls were included. This study was approved by the Institutional Review Board of Daegu Catholic University Medical Center. Written informed consent was obtained from all participants.

Measurement of circulating vitamin D levels: Participants provided blood samples between January and February 2013. Blood samples were centrifuged and sent on ice to the Neodin Medical Institute (Seoul, South Korea). Concentrations of serum 25(OH)D were measured using the DiaSorin radioimmunoassay (RIA) method at Neodin Medical Institute. The intra-assay coefficient of variation (CV) was less than 2%. All

laboratory technicians were blinded to the case status.

Assessment of lifestyle factors: We asked participants for information about their demographic characteristics, lifestyle factors, and family history of colorectal cancer. Dietary intake was assessed using a validated food frequency questionnaire (FFQ)[17]. The height and weight of participants were directly measured, and the body mass index (BMI) was calculated by dividing the weight in kilograms by the square of the height in meters. Participants were asked about the age at which they started and/or quit drinking and about the amount and frequency of alcohol consumption, such as rice wine (makgeolli), wine, beer, and liquor. Questions regarding cigarette smoking habits included queries about whether the participant smoked more than 20 packs of cigarettes, the age of smoking initiation and cessation, the amount of cigarettes smoked per day during regular smoking, and the total duration of regular cigarette smoking. The total pack years of smoking was calculated based on the total duration of regular cigarette smoking and the amount of cigarettes smoked per day during regular smoking. The metabolic equivalent of task (MET)-hours per week was calculated for physical activity.

Statistical analysis: The characteristics of the participants were compared between cases and controls using the means and standard deviations (SDs) for continuous variables or using the frequencies and percentages for categorical variables. The differences between continuous variables were analyzed by paired t-tests, and those between categorical variables were analyzed using the Mantel-Haenszel test. We used conditional logistic regression analysis to obtain the odds ratios (ORs) and 95% confidence intervals (CIs) of colorectal adenoma according to the quartile of the 25(OH)D levels. A test for trends was performed by including the median of each 25(OH)D quartile as a continuous variable. We adjusted for age (years, continuous), BMI (kg/m^2, 18.5-<23, 23-<25, > 25), alcohol drinking (men: nondrinker, past drinker, $\leqslant$ 1/mo, 2-4/mo, 2-3/wk, $\geqslant$ 4/wk, women: nondrinker, ever drinker), smoking status (men: never, 0-<20, 20-<30, > 30 pack-years of smoking; women: never, ever smoker), folate intake (mcg/d, continuous), and menopausal status and hormone replacement use for women only (premenopausal, postmenopausal without hormone replacement therapy, postmenopausal with hormone replacement therapy, postmenopausal with nonresponse about hormone replacement therapy). We examined whether the associations differed by adenoma calcium intake (median, < 412.5, $\geqslant$ 412.5 mg/d). We used the likelihood ratio test (LRT) to test the null hypothesis that there was no interaction due to the potential effect factors of colorectal adenoma. All *P* values were two-sided, and $P < 0.05$ was considered to be statistically significant. All analyses were performed using SAS 9.3 (SAS Institute Inc., Cary, North Carolina).

Meta-analysis

Selection of studies: We searched the PubMed database for studies published through February 25, 2015. We used the following terms for a PubMed search restricted to articles reported in English-language journals: ("Vitamin D" OR "Calcifediol" OR "circulating 25(OH)vitamin D" OR "25-hydroxylvitamin D" OR "25-hydroxyvitamin D" OR "25(OH)D") AND ("colorectal adenoma" OR "adenomas" OR "adenomatous" OR "CRA"). We also searched the Web of Science database using the term (25 hydroxyvitamin D and colorectal adenoma) in a search query of the topic field. In total, 203 articles were identified in the PubMed database and 55 articles in Web of Science. The title and abstract of each selected paper were examined in detail to determine whether the article was relevant. We also manually searched the bibliographies of the retrieved articles. The major criteria were as follows: (1) serum or plasma 25(OH)D was assayed as the factor of interest; (2) the outcome of interest was colorectal adenoma or adenoma recurrence; (3) the relative risk (RR) and 95%CIs were reported; and (4) articles were published as full-text manuscripts. If studies were duplicated[18-21], the study with the larger sample size[19] or a pooled analysis with another study[20] was included. Eligibility criteria were assessed by Choi YJ, and selected manuscripts were checked by an independent author (Lee JE). Two authors (Choi YJ and Lee JE) independently assessed the quality of each study using the Newcastle-Ottawa Scale[22]. Score differences greater than 1 between the two authors were resolved by consensus. We identified fifteen studies[13-15,19,20,23-31] that examined serum or plasma 25(OH)D levels and first colorectal adenoma or adenoma recurrence (Figure 1). We excluded study where the units of the 25(OH)D levels were not available[31]. The following data were extracted from the selected articles: the first author, published year, study region, sex, study design, endpoint, type of endoscopy, study dates (follow-up duration), number of cases and controls, mean or median of 25(OH)D, 25(OH)D levels comparing the highest category with the lowest category, OR (95%CI), and adjusted covariates. This meta-analysis was performed according to the Meta-analysis of Observational Studies in Epidemiology (MOOSE) guidelines[32].

Statistical analysis: For a meta-analysis of the association between 25(OH)D levels and colorectal adenoma, including the association found in our case-control study, we computed the summary RR and 95%CIs using a random-effects model[33]. The RR of each study was extracted from the most fully

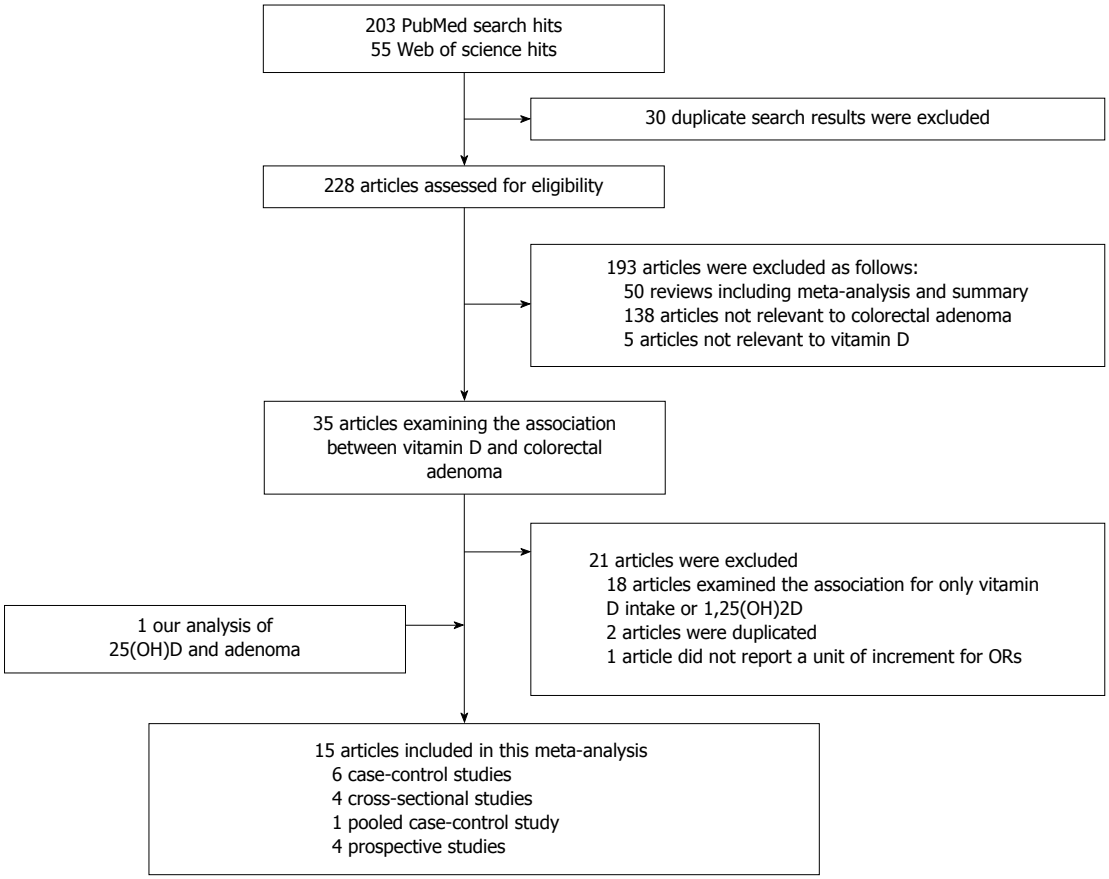

Figure 1 Flow chart of study selection process.

adjusted models if available. Estimates of the studies were weighted by the inverse of their variance. In the main analysis, we compared the highest category with the lowest category of circulating 25(OH)D levels. For a study that reported only a dose-response relationship[26], we calculated the OR (95%CI) of a mean difference in 25(OH)D levels between the highest and the lowest categories in the other studies for categorical comparison. We also constructed a dose-response model. If the RR per standard unit of increase was not presented in the studies, we converted the categorical RR to a dose-dependent RR using the method suggested by Greenland *et al*[34] and Orsini *et al*[35]. For this analysis, we assigned the midpoint of the upper and lower levels in each category. If the highest or the lowest boundary was not reported, we assumed that the interval in the highest or the lowest category had the same amplitude as the adjacent category. We calculated the RRs and 95%CIs for 10 ng/mL increments in the 25(OH)D levels.

We performed subgroup analyses and meta-regression analyses to assess potential sources of heterogeneity due to sex, calcium intake (high or low), geographic location (United States or Asia), or adenoma location (proximal or distal).

The between-study heterogeneity was evaluated using a *Q* test[33]. We evaluated for a potential publication bias using a funnel plot and Egger linear regression test[36]. All meta-analyses were performed using STATA 11 statistical software (StataCorp, College Station, TX, United States). All *P* values were two-sided, and $P < 0.05$ was considered to be statistically significant.

RESULTS

Case-control study

The characteristics according to colorectal adenoma prevalence are presented in Table 1. The mean age was 60.3 (SD = 5.3) years for the cases and 59.7 (SD = 5.4) years for the controls. The waist circumference and BMI were higher among the cases than among the controls. The cases consumed a higher amount of alcohol than did the controls. The mean 25(OH)D levels were 15.7 (SD = 5.8) ng/mL for the cases and 16.7 (SD = 5.6) ng/mL for the controls. Overall, 81.3% of the cases and 73.2% of the controls had 25(OH)D levels < 20 ng/mL. In our study, 76.3% of the men and 78.2% of the women had 25(OH)D levels < 20 ng/mL.

We found no association between the 25(OH)D levels and colorectal adenoma for men, but we found a suggestive inverse trend for women (Table 2); the multivariate ORs and 95%CIs compared to the bottom

Table 1 Characteristics of patients according to adenoma case, *n* (%)

	Adenoma (*n* = 112)	No adenoma (*n* = 112)	*P* value
Age (yr), mean ± SD	60.3 ± 5.3	59.5 ± 5.4	0.37
Men	57 (50.9)	57 (50.9)	Matched
25(OH)D (ng/mL), mean ± SD	15.7 ± 5.8	16.8 ± 5.6	0.16
25(OH)D < 20 ng/mL	91 (81.3)	82 (73.2)	0.12
Education			0.54
Elementary school graduate	16 (14.3)	10 (9.1)	
Middle school graduate	25 (22.3)	31 (28.2)	
High school graduate	50 (44.6)	46 (41.8)	
College graduate or above	21 (18.8)	23 (20.9)	
Waist circumference (cm), mean ± SD	87.0 ± 7.8	84.6 ± 6.7	0.02
BMI (kg/m^2)			0.10
18.5 < BMI < 23	33 (29.5)	36 (32.1)	
23 ≤ BMI < 25	32 (28.6)	45 (40.2)	
25 ≤ BMI	47 (41.9)	31 (27.7)	
Family history			0.53
Yes	6 (5.4)	4 (3.6)	
No	106 (94.6)	108 (96.4)	
Smoking status			0.29
Non smoker	56 (50.9)	64 (57.7)	
Past smoker	37 (33.6)	31 (27.9)	
Current smoker	17 (15.5)	16 (14.4)	
Alcohol drinking status			0.002
Never drinker	41 (36.6)	57 (50.9)	
Past drinker	6 (5.4)	6 (5.4)	
Current drinker	65 (58.0)	49 (43.8)	
Physical activity (MET-hr/wk), mean ± SD	29.0 ± 32.0	24.3 ± 25.2	0.22
Supplement use			0.08
Yes	42 (37.5)	56 (50.0)	
No	70 (62.5)	56 (50.0)	
Red meat intake			0.34
≤ 1/mo	11 (10.1)	10 (8.9)	
2-4/mo	77 (70.6)	88 (78.6)	
≥ 2/wk	21 (19.3)	14 (12.5)	
Energy intake (kcal/d), mean ± SD	1688.8 ± 581.9	1692.4 ± 488.4	0.96

P values were calculated using the Mantel-Haenszel test for categorical variables and the paired *t* test on loge-transformed variables. BMI: Body mass index.

quartile of 25(OH)D levels were 0.89 (0.26-3.10) for the 2nd quartile, 0.54 (0.13-2.16) for the 3rd quartile, and 0.22 (0.04-1.15) for the 4th quartile (*P* for trend = 0.05). When we stratified our data by adenoma location (proximal or distal), the ORs (95%CIs) were 0.55 (0.22-1.42) for distal adenomas and 0.59 (0.23-1.54) for proximal adenomas. Stratification by calcium intake showed 0.71 (0.19-2.58) for low calcium intake (less than median levels) and 0.36 (0.10-1.30) for high calcium intake (median or greater levels) of ORs (95%CIs) comparing the 4th quartile with the other three lower quartiles, but the difference was not statistically significant (*P* for interaction = 0.36).

Meta-analysis

A total of 15 articles reporting 5454 colorectal adenomas and 6656 controls were included in the meta-analysis (Table 3). The 25(OH)D levels were measured at baseline during clinical trials of colorectal adenoma recurrence[20,26]. As a result, we included 6 case-control studies, 4 cross-sectional studies, 1 pooled case-control study, and 4 prospective studies (two used a clinical trial design[20,26]). Ten studies were conducted in the United States, 1 in Austria, 2 in Japan, and 2 in Korea. Nine studies used the first adenoma as the endpoint, and two prospective studies from clinical trials considered adenoma recurrence as the endpoint. The other two studies[25], including our study, included a small proportion of participants who had recurrence. The endpoint of the study was not clear in one article[30]. Adenomatous polyps were determined through colonoscopy except in four studies; two studies used both colonoscopy and sigmoidoscopy[23,25], and the other two studies used only sigmoidoscopy[24,27]. Two studies conducted pooled analyses; the pooled analysis including three colonoscopy-based case-control studies (the Cancer Prevention Research Unit (CPRU), the Markers of Adenomatous Polyps (MAP) studies in North Carolina and the MAPII study in South Carolina)[28] and a pooled analysis of two randomized clinical trials (the Wheat Bran Fiber Trial and the Ursodeoxycholic Acid Trial)[20]. Out of 15 studies, 7 studies adjusted for the month of blood draw[14,19,25,27,29,30,37], one study matched the case and control by date of blood draw[23], and two Korean studies[15], including our case-control study, collected blood samples only in the winter.

When we conducted a meta-analysis of all the studies, we found an inverse association between the 25(OH)D levels and colorectal adenoma; the combined RR (95%CI) was 0.68 (0.54-0.82) when comparing the highest category with the lowest category (Figure 2). Because we found heterogeneity across studies (*P* < 0.001), we omitted one study at a time to examine whether one study influenced the overall results. When we excluded two studies[13,26] from the analysis, the heterogeneity decreased, but the results were similar to those from the analysis where we included all the studies. When we calculated a dose-response relationship, the combined RR (95%CI) was 0.93 (0.89-0.97) for a 10 ng/mL increment in 25(OH)D levels. When we limited our meta-analysis to the studies that included only the first colorectal adenoma, the combined RR (95%CI) was 0.65 (0.52-0.78) when comparing the highest category with the lowest category. We investigated the association between 25(OH)D levels and colorectal adenoma according to sex, study region, calcium intake, or adenoma site (Table 4). Stratification by study region showed that the ORs (95%CIs) were 0.70 (0.52-0.88) in the US and 0.66 (0.34-0.97) in Asia. The associations did not vary by these factors.

DISCUSSION

In a case-control study of Korean adults, we found

Table 2 Odds ratios and 95% confidence intervals for adenoma according to quartile of 25-hydroxyvitamin D level

	Quartile of serum 25(OH) D levels				
	Quartile1	Quartile2	Quartile3	Quartile4	*P* for trend
All patients					
Median (ng/mL)	10.33	14.25	17.91	24.21	
Case/control	37/28	26/28	28/28	21/28	
Model 1	1.00	0.71 (0.35-1.45)	0.73 (0.33-1.61)	0.52 (0.23-1.20)	0.15
Model 2	1.00	0.72 (0.31-1.66)	0.73 (0.29-1.82)	0.49 (0.19-1.27)	0.16
Men					
Median (ng/mL)	11.08	14.6	17.65	22.66	
Case/control	15/14	13/14	17/15	12/14	
Model 1	1.00	0.95 (0.35-2.57)	1.10 (0.29-4.18)	0.80 (0.25-2.60)	0.74
Model 2	1.00	1.31 (0.36-4.82)	1.26 (0.23-6.87)	1.80 (0.40-8.12)	0.46
Women					
Median (ng/mL)	9.48	13.53	18.4	25.42	
Case/control	16/14	22/14	8/14	9/13	
Model 1	1.00	1.31 (0.53-3.24)	0.51 (0.18-1.51)	0.65 (0.20-2.14)	0.16
Model 2	1.00	0.89 (0.26-3.10)	0.54 (0.13-2.16)	0.22 (0.04-1.15)	0.05

Model 1 was adjusted for age (continuous). Model 2 was adjusted for the following covariates; men: age (years, continuous), body mass index (BMI) (kg/m^2, 18.5- < 23, 23- < 25, > 25), alcohol drinking (nondrinker, past drinker, ⩽ 1/mo, 2-4/mo, 2-3/wk, ⩾ 4/wk), smoking status (never, 0- < 20, 20- < 30, > 30 pack-years of smoking), and folate intake (mcg/d, continuous); women: age (years, continuous), BMI (kg/m^2, 18.5- < 23, 23- < 25, > 25), alcohol drinking (nondrinker, ever drinker), smoking status (never, ever smoker), folate intake (mcg/d, continuous), and menopausal status and hormone replacement use (premenopausal, postmenopausal without hormone replacement therapy, postmenopausal with hormone replacement therapy, postmenopausal with nonresponse about hormone replacement therapy use).

Table 3 Included studies of circulating levels of 25-hydroxyvitamin D and colorectal adenoma

First author, year	Country (sex)	Study design	study dates	Endpoint	Type of endoscopy	25(OH)D, mean or median	No. of cases/ controls	25(OH)D levels in the highest *vs* lowest categories	OR (95%CI)
Platz, 2000	United States (W)	Prospective study	1989-1996	First adenoma	Sigmoidoscopy or colonoscopy	26.4 in cases and 26.8 ng/mL in controls, mean	326/326	38.0 ng/mL *vs* 16.3 ng/mL, median	1.00 (ref), 0.64, 0.58, 1.04 (0.66-1.66)
Levine, 2001	United States (M, W)	Case-control study	1991-1993	First adenoma	Sigmoidoscopy	25.6 in cases and 26.9 ng/mL in controls, mean	473/506	34.3-115 ng/mL *vs* 1-15.2 ng/mL, range	1.00 (ref), 0.99, 0.86, 0.74 (0.51-1.09)
Peters, 2001	United States (M, W)	Case-control study	1994-1996	First (61%) or recurrent adenoma	Colonoscopy (86.2%) or sigmoidoscopy	24.7 in cases and 26.5 ng/mL in controls, median	236/218	33.7-67.2 ng/mL *vs* 5.3-19.4 ng/mL, range	1.0(ref), 0.40, 0.67, 0.47, 0.43(0.23-0.81)
Grau, 2003	United States (M, W)	Prospective study	1992-1996	Recurrent adenoma	Colonoscopy	29.1 ng/mL, median	376/422	-	0.99 (0.91-1.07) OR for serum vitD levels per 12 (1SD) units increase
Peters, 2004	United States (M, W)	Prospective study	1993-1999	First advanced adenoma	Sigmoidoscopy	27.0 in cases and 28.3 ng/mL in controls, mean	394/397	-	0.87 (0.75-1.01) OR for serum vitD levels per 10 units increase.
Miller, 2007	United States (M, W)	Cross-sectional study	1998-2000	First adenoma	Colonoscopy	27.5 in cases and 31.4 ng/mL in controls, mean	111/238	> 33.8 ng/mL *vs* < 20.8 ng/mL, range	1.00 (ref), 0.74, 0.51 (0.27-0.98)
Takahashi, 2010	Japan (M)	Case-control study	1997-2004	First adenoma	Colonoscopy	26.2 in cases and 26.1 ng/mL in controls, mean	656/648	⩾ 30 ng/mL *vs* < 22 ng/ml, range	1.00 (ref), 1.21, 1.21, 1.25 (0.85-1.84)

Fedirko, 2010	United States (M, W)	Pooled case-control study	1991-2002	First adenoma	Colonoscopy	24.5 in cases and 25.5 ng/mL in controls, mean	616/770		1.00 (ref), 0.77, 0.85, 0.59 (0.41-0.84)
Adams, 2011	United States (M, W)	Cross-sectional study	1998- 2003	Fitst or recurrent adenoma	Colonoscopy	23.1 in cases and 24.9 ng/mL in controls, mean	149/ 225	> 28.9 ng/mL *vs* ≤ 20.5 ng/ml, range	1.00 (ref), 0.97, 0.71 (0.38-1.30)
Ashktorab, 2011	United States (M, W)	Case-control study			Colonoscopy	41.2 in cases and 41.4 ng/mL in controls, mean	93/187	> 57.7 ng/mL *vs* < 29.5 ng/mL, range	1.00 (ref), 1.4, 1.9, 0.6 (0.3-1.4)
Hong, 2012	Korea (M, W)	Case-control study	2009-2010	First adenoma	Colonoscopy	20.0 in cases and 25.0 ng/mL in controls, mean	143/ 143	≥ 23.9 ng/mL *vs* < 14.3 ng/mL, range	1.00 (ref), 0.87, 0.40, 0.38 (0.18-0.80)
Yamaji, 2012	Japan (M,W)	Cross-sectional study	2004-2005	First adenoma	Colonoscopy		737/ 703	32 ng/mL *vs* 16 ng/mL, median	1.00 (ref), 0.86, 0.91, 1.03, 0.64 (0.45-0.92)
Jacobs, 2013	United States (M, W)	Prospective study	1990-1999	Recurrent adenoma	Colonoscopy		942/ 1132	> 30 ng/mL *vs* < 20 ng/mL, range	1.00 (ref), 0.91, 0.95 (0.73-1.24)
Aigner, 2014	Austria (W)	Cross-sectional study	2010-2013	First adenoma	Colonoscopy	22.8 ng/mL in women	90/629		0.976 (0.954-0.999) for 1 ng/mL increment
Choi, 2015 (our study)	Korea (M, W)	Case-control study	2011-2012	First or recurrent(5%) adenoma	Colonoscopy	15.7 ng/mL in cases and 16.6 in controls	112/112	23.4 ng/mL *vs* 10.0 ng/mL, mean	1.00 (ref), 0.72, 0.73, 0.49 (0.19-1.27)

Table 4 Combined relative risk (RR)*s* and 95% confidence intervals of colorectal adenoma for the associations by sex, calcium intake, study region, and adenoma site

	Studies, *n*	Ref.	Combined RR (95%CI) comparing the highest *vs* the lowest categories	*P* for difference
Sex				0.30
Men	6	[13-15,20,27] and our study	0.78 (0.51-1.06)	
Women	7	[14,15,20,23,27,37] and our study	0.58 (0.33-0.83)	
Calcium intake				0.48
Low	7	[14,19,24-26,28] and our study	0.73 (0.46-1.01)	
High	7	[14,19,24-26,28] and our study	0.61 (0.40-0.82)	
Study region				0.88
United States	10	[19,21,23-30]	0.70 (0.52-0.88)	
Asia	4	[13-15] and our study	0.66 (0.35-0.98)	
Site				0.50
Distal adenoma	10	[13-15,21,23-25,27,28] and our study	0.67 (0.53-0.81)	
Proximal adenoma	8	[13-15,21,25,28,37] and our study	0.61 (0.44-0.79)	

an inverse association between circulating serum 25(OH)D levels and colorectal adenoma in women but not in men. When we combined estimates from case-control, cross-sectional or prospective studies in a meta-analysis, higher circulating vitamin D levels were associated with a lower prevalence of colorectal adenoma. The associations were similar across proximal and distal sites. Notably, our meta-analysis showed a significant inverse association between 25(OH)D levels and colorectal adenoma in Asian populations.

We found an inverse association only among women in our case-control study. We cannot rule out the possibility that our finding could be attributed to chance or potential residual confounding factors among men; however, the stronger association among women compared with that in men in our meta-analysis and the stronger, or only significant for women, association in some studies[21,27] warrant further studies.

Although we found an inverse association between

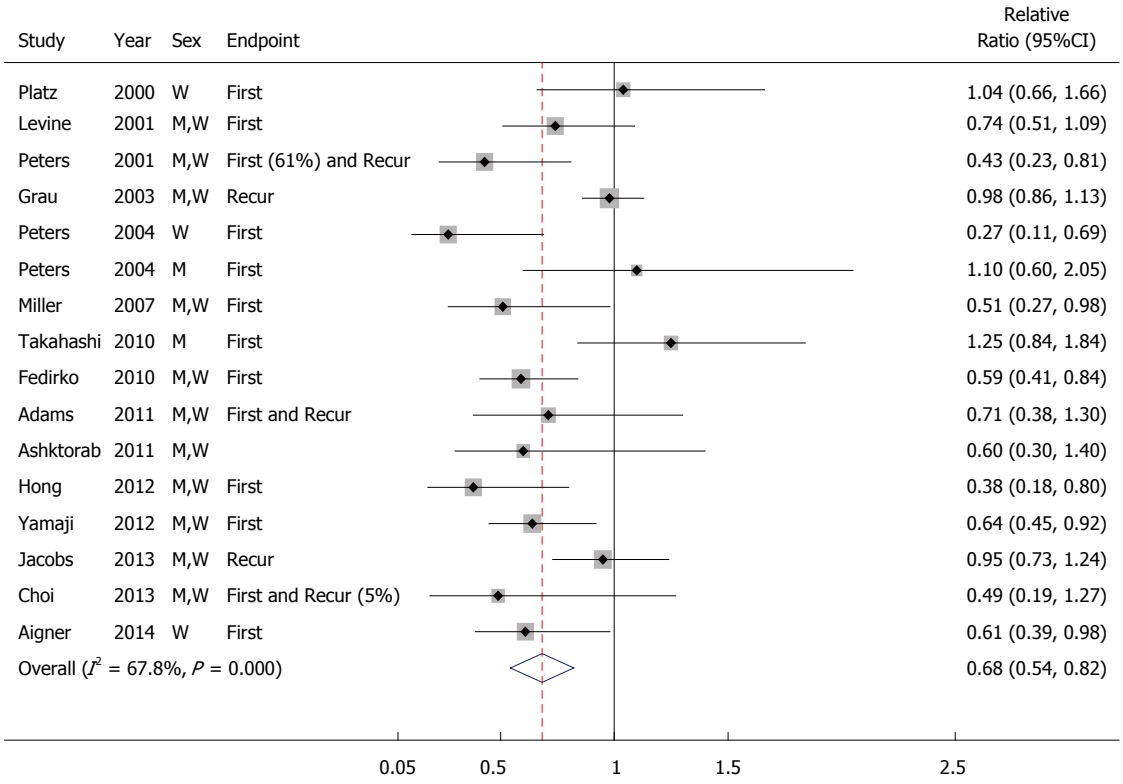

Figure 2 Study-specific and combined odd ratios and 95%CIs of colorectal adenoma comparing the highest category with the lowest category of circulating 25-hydroxyvitamin D levels. First: First adenoma as an endpoint; Recur: Recurrent adenoma as an endpoint.

circulating vitamin D levels and colorectal adenoma in a previous meta-analysis[16], a limited number of Asian studies did not allow us to explore whether high vitamin D was associated with a lower prevalence of colorectal adenoma in Asian populations along with Western populations. Because more studies have been published and we added our study, we examined the potential benefit of vitamin D against colorectal neoplasia in Asian populations. It needs further prospective studies.

Because vitamin D and calcium are metabolically interrelated[38], we examined whether the association between 25(OH)D levels and colorectal adenoma varied by calcium intake in the meta-analysis. Although we found a stronger association among those with high calcium intake than those with low intake, the difference was not statistically significant.

Experimental studies have shown that $1,25(OH)_2D$ inhibits cellular proliferation, induces differentiation and apoptosis, and inhibits angiogenesis[39,40]. In an *in vitro* study, colon tumor tissues expressed a lower vitamin D receptor level than did normal tissues, and the tumor with a higher receptor level was more responsive to $1,25(OH)_2D$[41]. Additionally, the administration of $1,25(OH)_2D$ or vitamin D analogues induced the expression of genes involved in cell differentiation[42,43]. In an *in vivo* study, vitamin D treatment in Wistar rats reduced the apoptosis in colon tumors[44].

Our study has several strengths and limitations. We collected blood samples only during the winter season; therefore, individual seasonal variations should not contribute to our findings. We performed a comprehensive meta-analysis to combine existing evidence and found an inverse association between 25(OH)D levels and colorectal adenoma. The limitations of our case-control study include the small sample size, the single measurement of 25(OH)D levels, and the possibility of the presence of residual confounding factors. Also, because our study participants provide blood samples after colonoscopy, vitamin D levels could have been changed if participants altered their lifestyle such as outdoor activities and dairy food intake. Especially, if vitamin D levels in participants with adenoma increased, the association would have been attenuated toward no association. However, an inverse association observed in a meta-analysis may suggest that an inverse association in women in our case-control study may not be a seriously biased result. We cannot rule out the possibility that no association in men could be partly due to the limitations of retrospective nature in our study.

In summary, the results from our case-control study and meta-analysis showed that circulating 25(OH)D levels are inversely associated with the prevalence of colorectal adenoma in both Western and

Asian populations.

COMMENTS

Background

Low sunlight exposure, partly due to sedentary lifestyles and lack of outdoor activities, has been suggested to contribute to colorectal cancer development, and the role of vitamin D in colorectal cancer prevention has drawn increasing attention.

Research frontiers

The authors conducted a case-control study in a Korean population and calculated a summary estimate through a meta-analysis to examine the association between circulating 25-hydroxyvitamin D [25(OH)D] levels and colorectal adenoma.

Innovations and breakthroughs

This study suggest an inverse association between circulating 25(OH)D levels and colorectal adenoma in both Western and Asian populations.

Applications

This study suggests that vitamin D may exhibit a protective effect against the early stages of colorectal neoplasia.

Terminology

25(OH)D is hydroxylated in the kidney to form 1,25-dihydroxyvitamin D. $1,25(OH)_2D$, a biologically active form of vitamin D, is responsible for most biologic functions. Circulating concentration of 25(OH)D is known to be a good reflection of exposure to sunlight and dietary intake of vitamin D.

Peer-review

This is a well written manuscript in writing and structure. The authors performed a case-control study among Korean adults determining the association between colorectal adenoma and 25(OH)D levels, moreover they systematically summarized the studies that have been performed previously.

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ORIGINAL ARTICLE

Retrospective Study

Value of two-phase dynamic multidetector computed tomography in differential diagnosis of post-inflammatory strictures from esophageal cancer

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Conflict-of-interest statement: There is no conflict of interest.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at sburyakina@yandex.ru. The presented data are anonymized and risk of identification is low. No additional data are available.

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Abstract

AIM: To characterize the computed tomography (CT) findings in patients with post-inflammatory esophageal strictures (corrosive and peptic) and reveal the optimal scanning phase protocols for distinguishing post-inflammatory esophageal stricture and esophageal cancer.

METHODS: Sixty-five patients with esophageal strictures of different etiology were included in this study: 24 patients with 27 histopathologically confirmed corrosive strictures, 10 patients with 12 peptic strictures and 31 patients with esophageal cancer were evaluated with a two-phase dynamic contrast-enhanced MDCT. Arterial and venous phases at 10 and 35 s after the attenuation of 200 HU were obtained at the descending aorta, with a delayed phase at 6-8 min after the start of injection of contrast media. For qualitative analysis, CT scans of benign strictures were reviewed for the presence/absence of the following features: "target sign", luminal mass, homogeneity of contrast medium uptake, concentric wall thickening, conically shaped

suprastenotic dilatation, smooth boundaries of stenosis and smooth mucous membrane at the transition to stenosis, which were compared with a control group of 31 patients who had esophageal cancer. The quantitative analysis included densitometric parameter acquisition using regions-of-interest measurement of the zone of stenosis and normal esophageal wall and the difference between those measurements (ΔCT) at all phases of bolus contrast enhancement. Esophageal wall thickening, length of esophageal wall thickening and size of the regional lymph nodes were also evaluated.

RESULTS: The presence of a concentric esophageal wall, conically shaped suprastenotic dilatation, smooth upper and lower boundaries, "target sign" and smooth mucous membrane at the transition to stenosis were suggestive of a benign cause, with sensitivities of 92.31%, 87.17%, 94.87%, 76.92% and 82.05%, respectively, and specificities of 70.96%, 89.66%, 80.65%, 96.77% and 93.55%, respectively. The features that were most suggestive of a malignant cause were eccentric esophageal wall thickening, tuberous upper and lower boundaries of stenosis, absence of mucous membrane visualization, rupture of the mucous membrane at the upper boundary of stenosis, cup-shaped suprastenotic dilatation, luminal mass and enlarged regional lymph nodes with specificities of 92.31% 94.87%, 67.86%, 100%, 97.44%, 94.87% and 82.86%, respectively and sensitivities of 70.97%, 80.65%, 96.77%, 80.65%, 54.84%, 87.10% and 60%, respectively. The highest tumor attenuation occurred in the arterial phase (mean attenuation 74.13 ± 17.42 HU), and the mean attenuation difference between the tumor and the normal esophageal wall (mean ΔCT) in the arterial phase was 23.86 ± 19.31 HU. Here, 11.5 HU of ΔCT in the arterial phase was the cut-off value used to differentiate esophageal cancer from post-inflammatory stricture (P = 0.000). The highest attenuation of post-inflammatory strictures occurred in the delayed phase (mean attenuation 71.66 ± 14.28 HU), and the mean ΔCT in delayed phase was 34.03 ± 15.94 HU. Here, 18.5 HU of ΔCT in delayed phase was the cut-off value used to differentiate post-inflammatory stricture from esophageal cancer (P < 0.0001).

CONCLUSION: The described imaging findings reveal high diagnostic significance in the differentiation of benign strictures from esophageal cancer.

Key words: Multidetector computed tomography; Esophageal cancer; Corrosive stricture; Peptic stricture

Core tip: Two-phase dynamic multidetector computed tomography was proposed to evaluate esophageal stenosis. No previous studies have evaluated the utility of this method for post-inflammatory strictures. We investigated this method's ability to evaluate benign strictures by qualitatively and quantitatively assessing changes in the esophageal walls and demonstrated that the majority of patients with benign strictures had concentric wall thickening with smooth boundaries, conically shaped suprastenotic dilatation, a "target sign", smooth mucous membrane at the transition to stenosis. An assessment of the dynamics of contrast material accumulation by strictures revealed that the arterial and delayed phases are optimal for differentiating benign strictures from esophageal cancer.

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INTRODUCTION

Although computed tomography (CT) has not been used as the primary modality for evaluating esophageal lesions, both benign and malignant stenoses may be manifested on CT by focal or extended thickening of the esophageal wall[1,2]. Other causes of a thickened esophageal wall on CT include Barrett's esophagus, esophagitis, secondary achalasia, diffuse esophageal spasm, varices, and esophageal intramural pseudodiverticulosis[3]. Some characteristic CT-findings of peptic stenosis and corrosive esophageal strictures have been briefly described in the literature[4-6]. To our knowledge, no detailed description of CT findings in post-inflammatory benign esophageal strictures has been published in the radiology literature. No study has analysed the value of CT with bolus contrast enhancement in the differential diagnosis of esophageal stenosis with a benign etiology. The purpose of our study was to assess the significantly more common CT findings in patients with post-inflammatory benign esophageal strictures (corrosive and peptic) and thereby reveal the optimal scanning phases of the protocol for distinguishing post-inflammatory esophageal stricture and esophageal cancer.

MATERIALS AND METHODS

Patient characteristics

After examining the PACS database for the period from October 2010 to December 2014, 65 patients, who underwent thoracic CT and had a definitive diagnosis, were included in this retrospective study. A review of the patients' medical records and CT reports identified 31 patients (25 men, 6 women; mean age 65 years; range, 41-81 years) with a diagnosis of esophageal cancer, 24 patients (11 men, 12 women; mean age 48 years; range, 23-74 years) with 27

corrosive esophageal strictures, and 10 patients (6 men, 4 women; mean age, 49 years; range, 19-66 years) with 12 peptic strictures. The cause of stenosis included ingestion of caustic agents in 23 patients, and gastroesophageal reflux disease in 10 patients. In the 31 other patients a clinical diagnosis of esophageal cancer was made by esophagography or endoscopy and clinical data. Patients with stents, after radiotherapy and chemotherapy were not included in the study because pronounced fibrous tissue around the stent and necrotic areas in the tumor may affect the MDCT image of a typical stenosis tissue.

The definitive pathologic diagnosis was based on the results of biopsy in 5 cases of T4 stage esophageal cancer, the results of endoscopy in 3 cases with short corrosive esophageal strictures and in 5 cases of peptic strictures. The patients with T4 stages esophageal cancer underwent palliative treatment, and patients with those with strictures had sessions of bougienage. Another 57 patients underwent surgery, which comprised: either transthoracic and/or transhiatal esophagectomy with posterior mediastinal gastric tube or replacement of the left part of the colon. All pathologic specimens were histologically investigated after surgery by a pathologist. The histologic assessment of benign *vs* malignant stenosis was determined based on the standard architectural and cytologic features[7,8].

CT

CT scans were obtained as part of the initial assessment of these patients at our institution. Multidetector CT (MDCT) was performed using 64 and 256 MDCTs (Philips Brilliance CT-64, Brilliance iCT-256 (Philips Medical Systems (Cleveland, Ohio 44143 United States). Examination was performed in the supine position with patient's hands behind his/her head. Scanning was performed with a gulp of potable water in unenhanced, arterial and delayed phases with the patient holding their breath. Cranio-caudal scanning from neck to upper abdomen was performed. The following scanning parameters were used: collimation 0.9 mm, reconstruction interval 0.45 mm, pitch 1, tube rotation rate 0.75 s. Nonionic contrast agents (Optiray 350, Omnipaque 350, Ultravist 370, Visipaque 320) were injected intravenously using a dual head automatic injector OptiVantage DH (Mallinckrodt; InC) with a rate of 4-5 mL/s. A bolus of contrast agent was followed by bolus "chaser" (normal saline, 40-50 mL, with the same rate).

To start scanning, the "bolus tracking" software package was used. Two contrast enhanced phases were performed at 10 and 34 s for arterial and venous phases after the attenuation of the descending aorta reached 200 HU. The delayed phase was performed 4-6 min after the injection of contrast agent.

All data were reconstructed with a 1 mm section thickness at 1-mm intervals. Post-processing was performed using the Brilliance Portal [Philips Medical Systems (ClevelanD)]. Images were assessed in all examination phases.

Image analysis

The thoracic CT of 64 patients was reviewed retrospectively by two experienced radiologists, who had 4 and 6 years of experience in gastrointestinal radiology. All images were de-identified. Radiologists were blinded to the histological results, numbers and locations of the stenoses described in the surgical, radiologic and endoscopic findings. The final interpretations were made by consensus.

All CT data were assessed for the following findings: (1) thickness of the esophageal wall (concentric or eccentric); (2) upper and lower boundaries at the coronal view of the multiplanar reconstruction image (smooth or tuberous); (3) a luminal mass (presence or absence); (4) a "target sign" (presence or absence); (5) contrast medium uptake by thickened esophageal walls (heterogeneity or homogeneity); (6) suprastenotic dilatation (cup-shaped or conically shaped); (7) mucous membrane at the transition to stenosis (smooth or rupture); and (8) thickness, length and size of the regional lymph nodes (mm).

The esophageal wall was considered thickened when its thickness was greater than 5 mm[1,9]. The actual wall thickness and length of thickened walls in these patients were measured (mm). Wall thickening was considered eccentric when there was asymmetry in the thickening of the two walls of the esophagus. When the lumen was not obliterated or partially collapsed, the thickness of a single esophageal wall was measured from its outer to inner borders. In the patients in whom the lumen could not be identified (obliterated or collapsed), thickness was calculated as one half of the thickness, measured from the outer border of one wall to the outer border of the opposing wall in the short axis of the cross section of the esophagus.

The upper and lower boundaries of thickened walls were analysed at the coronal view of the multiplanar reconstruction image and were considered to be tuberous if they were irregular.

A luminal mass was considered to be present when there was a soft-tissue mass in the lumen that arose from the esophageal wall.

A "target sign" was considered to be present when there was a combination of an enhanced saved mucosa and a hypodense submucosa in the thickened esophageal walls[1,10].

Heterogeneity of contrast medium uptake was considered to be present when hypo- and/or hyperdense components were visualized in pathologically changed esophageal walls.

If a suprastenotic dilatation was present, the radiologist noted whether it was conically shaped or

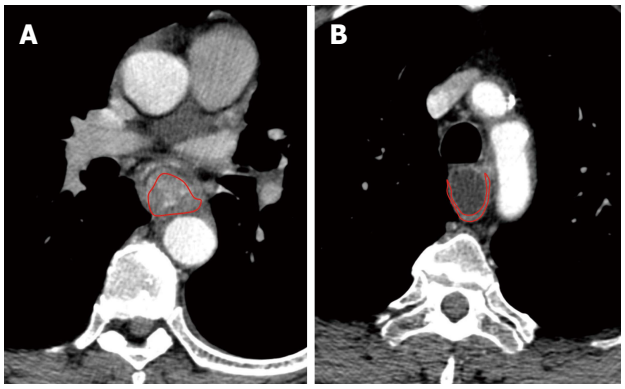

Figure 1 Visualization of reliable region of interest. A: Regions of interest (circled red) for esophageal stenosis were drawn freehand around the thickened walls; B: Regions of interest (circled red) for the background intact esophagus were drawn around the intact esophageal wall.

cup-shaped.

The shape of a mucous membrane at the transition to stenosis at the coronal view of the multiplanar reconstruction image was analysed, concerning whether it was smooth or whether its rupture occurred at the upper boundary of stenosis.

In all cases a short-axis diameter of the largest regional (cervical, tracheobronchial, mediastinal, gastric or celiac) lymph node was analyzed. It was considered enlarged if its short-axis diameter was greater than 10 mm in the transverse plane.

Esophageal wall attenuation in hounsfield units (HU) was measured at all phases of bolus contrast enhancement. A reliable region of interest (ROI) within the thickened esophageal wall was manually drawn in the transverse section, and the area of ROI was more than 60% of that of the entire thickened esophageal wall in the same section (Figure 1). The tumor attenuation value was derived automatically by the workstation software. To minimize partial volume averaging with the surrounding tissues, the intraluminal gas and periesophageal fat were carefully excluded when drawing the ROI of stenosis. The attenuation of the normal esophagus in groups was measured by drawing the ROI of the intact esophageal walls. Subsequently, ΔCT was calculated by subtracting the referenced attenuation value of the background normal esophageal wall from the representative attenuation value for esophageal stenosis.

Statistical analysis

Patients were divided into two groups based on the pathologic findings. Patients with histopathologically confirmed corrosive (n = 27) and peptic (n = 12) strictures were included in group A ("benign stenosis") and patients with esophageal cancer (n = 31) were included in group B ("malignant stenosis").

Correlations between the features and groups A and B were examined. We used the Pearson product moment correlation coefficient (r) to assess the strengths of the associations that involve normal data distributions of quantitative features and the Spearman's rank correlation test (r_s) to assess the strengths of the associations that involve non-normal data distributions of quantitative CT features and qualitative CT features. A of P value less than 0.05 was considered statistically significant. The general classification of correlations according to their strength was as follows: (1) strong, or close when the correlation coefficient was more than 0.70; (2) average from 0.50 to 0.69; (3) moderate from 0.30 to 0.49; (4) weak from 0.20 to 0.29; and (5) weakest less than 0.19. CT features with strong and average values of the correlation coefficient were suggested to be the most useful for differentiating post-inflammatory benign esophageal strictures and esophageal cancer. Sensitivity and specificity were also calculated for all features.

With statistical software (version 17.0 for Windows, SPSS), independent sample Student's t tests were performed to compare ΔCT between the groups with esophageal cancer and the groups with post-inflammatory strictures. A P value less than 0.05 indicated a significant difference. After a significant difference was shown, receiver operating characteristic (ROC) analysis was carried out to determine the cut-off of ΔCT for discriminating stenosis (esophageal cancer and post-inflammatory benign esophageal strictures) from background normal esophagus. Measurements obtained at the gastroesophageal junction were excluded from the determination of the overall mean wall thickness because of a known problem with apparent thickening of the wall or pseudomass lesions at this level[8,10]. The statistical methods of this study were reviewed by Margarita V. Khakhanova of the Vishnevsky Institute of Surgery.

RESULTS

The imaging findings obtained from groups A ("benign stenosis") which included 27 corrosive and 12 peptic strictures and B ("malignant stenosis"), which included 31 esophageal cancers, are summarized in Table 1. In the pathologic reports, esophageal squamous cell cancer was found in 23 cases and esophageal adenocarcinoma was found in 8 cases. Seventeen (54.8%) patients with esophageal cancer had T3 stage, 5 (16.2%) patients had T4 stage, 6 (19.4%) patients had T2 stage, and 3 (9.6%) patients had T1 stage, according to the classification of the Union for International Cancer Control.

The correlation analysis results examining the CT features in group A are summarized in Table 2.

In group A, esophageal thickening (more than 5 mm) was observed in 32 (82%) patients; in group B, esophageal thickening was observed in 30 (96.8%) patients. The overall mean esophageal wall thickness was 17.6 mm with a range of 6-38 mm (SD = 7.3 mm; 95%CI: 14.9-20.3 mm) in the 31 patients with malignant stenosis *vs* 8.68 mm with a range of 4-21 mm (SD = 3.4 mm; 95%CI: 7.56-9.81 mm) in the 39

Table 1 Computed tomography features of esophageal stenosis

CT features	Group A (benign stenosis, *n* = 39)	Group B (malignant stenosis, *n* = 31)
Luminal mass	2	27
Heterogeneity of contrast medium uptake	3	10
Homogeneity of contrast medium uptake	36	21
Eccentric esophageal wall thickening	3	22
Concentric esophageal wall thickening	37	9
Suprastenotic dilatation cup-shaped	1	17
Suprastenotic dilatation conically shaped	34	5
Smooth mucous membrane at the transition to stenosis	32	2
Rupture of the mucous membrane at the upper boundary of stenosis	0	25
Smooth upper and lower boundaries	37	6
Tuberous upper and lower boundaries	2	25
Presence of the mucous membrane in the stenosis «target sign»	30	1
Absence of the mucous membrane visualization in thickened walls	9	30
Presence of enlarged lymph nodes	6	18

CT: Computed tomography.

Table 2 Results of correlation analysis in group A (benign stenosis)

CT-features	Spearman's rank correlation test (r_s), Pearson product moment correlation coefficient (r)	*P* value
Concentric esophageal wall thickening	r_s = 0.656	0.000
Smooth upper and lower boundaries	r_s = 0.510	0.000
Absence of the luminal mass	r_s = 0.827	0.000
Presence of the mucous membrane in the stenosis ("target sign")	r_s = 0.779	0.000
Homogeneity of contrast medium uptake	r_s = 0.354	0.003
Conically shaped suprastenotic dilatation	r_s = 0.711	0.000
Smooth mucous membrane at the transition to stenosis	r_s = 0.657	0.000
Size of the regional lymph nodes	r_s =0.542	0.000
Wall thickness (mm)	r_s = 0.080	0.437
Length of esophageal wall thickness (mm)	r_s = 0.263	0.290
CT attenuation value of the post-inflammatory stricture in native phase	r = 0.055	0.652
CT attenuation value of the post-inflammatory stricture in arterial phase	r = -0.736	0.000
CT attenuation value of the post-inflammatory stricture in venous phase	r = -0.444	0.000
CT attenuation value of the post-inflammatory stricture in delayed phase	r = 0.579	0.000
ΔCT in unenhanced phase	r = 0.068	0.594
ΔCT in arterial phase	r = -0.709	0.000
ΔCT in venous phase	r = -0.565	0.000
ΔCT in delayed phase	r = 0.652	0.000

Computed tomography (CT) features that have strong and average correlation coefficient values are underlined.

patients with benign stenosis. However, the correlation between wall thickness and the type of stenosis was insignificant (r_s = 0.080, *P* = 0.437). The overall mean length of esophageal wall thickness was 71.33 mm with a range of 11-135 mm (SD = 33.67 mm; 95%CI: 58.76-83.91 mm) in the patients with malignant stenosis *vs* 56.57 mm, with a range of 3-350 mm (SD = 61.22 mm; 95%CI: 36.16-76.98 mm), in patients with benign stenosis. However, the correlation between the length and type of stenosis was also insignificant (r_s = 0.263, *P* = 0.29). In group A the mean size of the regional lymph nodes was 6.82 ± 2.9 (range, 3-15) and in group B it was 10.77 ± 3.6 (range, 5-18). An average correlation between enlarged regional lymph nodes and etiology of the stenosis was found (r = 0.542, *P* < 0.000).

The presence of the following factors was suggestive of a benign cause: concentric esophageal wall thickening with a sensitivity of 92.31% and a specificity of 70.96%; conically shaped suprastenotic dilatation, with a sensitivity of 87.17% and a specificity of 89.66%; smooth upper and lower boundaries, with a sensitivity of 94.87% and a specificity of 80.65%; presence of the mucous membrane in the stenosis, with a sensitivity of 76.92% and a specificity of 96.77%; and smooth mucous membrane at the transition to stenosis, with a sensitivity of 82.05% and a specificity of 93.55% (Figure 2). The correlation between the homogeneity of contrast medium uptake and the type of stenosis was moderate (r_s = 0.354, *P* = 0.003). This feature had a high sensitivity (92.31%) but low specificity (32.26%).

We identified the following imaging findings of malignant stenosis: eccentric esophageal wall thickening with a specificity of 92.31% and a sensitivity of 70.97%; tuberous upper and lower boundaries of stenosis with a specificity of 94.87% and a sensitivity of 80.65%; absence of the mucous membrane visualization in stenosis, which were observed in the majority of patients (96.8%) with a specificity of 67.86% and a sensitivity of 96.77%; rupture of the mucous membrane at the upper boundary of stenosis with a specificity of 100% and a sensitivity of 80.65%; cup-shaped suprastenotic dilatation with a specificity of 97.44% and a sensitivity of 54.84%; luminal mass with a specificity of 94.87% and a sensitivity of 87.10% and enlarged regional lymph nodes with a specificity of 82.86% and a sensitivity of 60%. All of these features were significantly more common in malignant than in benign esophageal stenosis (*P* < 0.05) (Figure 3).

Difference in CT values among esophageal cancer, post-inflammatory strictures and background normal esophagus

The correlation coefficient (r) between the CT attenuation value and group A was strong in the arterial phase (r = -0.736, *P* = 0.000) and average in the delayed phase (r = 0.579, *P* = 0.000), thus these features were suggested to be the most significant for differentiating benign esophageal

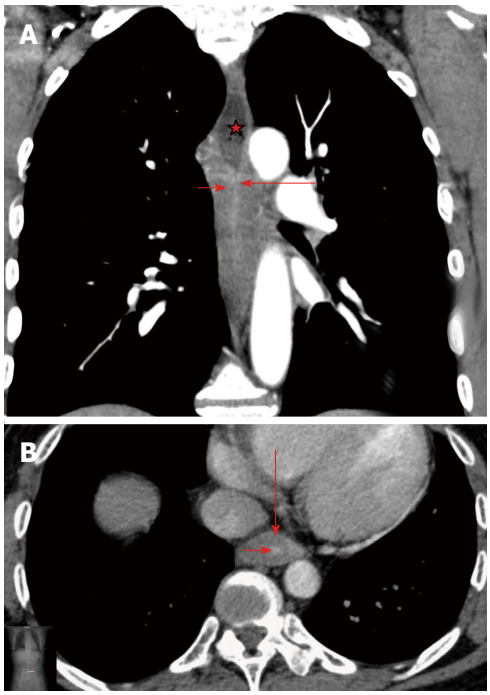

Figure 2 Corrosive esophageal stricture. A: Multidetector computed tomography (MDCT). Arterial phase. Coronal reconstruction. Concentric esophageal wall thickening of the corrosive stricture, which has a homogeneous structure (short arrow). The mucosa is traceable as a thin hyperintense line in the centre of thickened walls that was caused by fibrotic changes (long arrow). Conically shaped suprastenotic dilatation (star) with smooth upper boundaries of the stricture; B: MDCT. Arterial phase. Axial CT scan. Target sign - thickening of saved esophageal mucosa (short arrow) in the centre of fibrotically changed submucosal, muscular layers and adventitia of esophageal walls (long arrow).

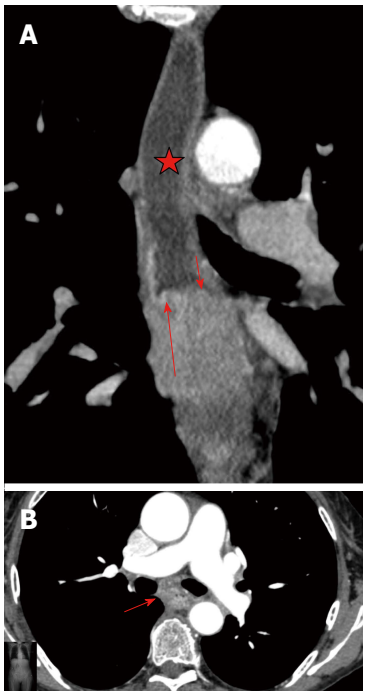

Figure 3 Esophageal cancer. A: Multidetector computed tomography (MDCT). Arterial phase. Coronal reconstruction of esophageal cancer. Tuberous upper and lower boundaries of stenosis (short arrow); rupture of the mucous membrane at the upper boundary of stenosis (long arrow); cup-shaped suprastenotic dilatation (star); B: MDCT. Arterial phase. Axial CT scan of esophageal cancer. Eccentric esophageal wall thickening without mucous membrane visualization in stenosis (arrow).

stricture and esophageal cancer. Due to the strong negative correlation observed in the benign group, the mean CT attenuation value in the arterial phase was calculated in the malignant group [74.13 ± 17.42 HU (range, 34-105 HU)]. In the arterial phase, the mean CT attenuation value of background intact esophagus was 42.45 ± 8.18 HU (range, 24-60 HU) in all patients in groups A and B. The mean ΔCT in the arterial phase was 23.86 ± 19.31 HU. To distinguish esophageal carcinoma from the background normal esophageal walls, an ROC curve analysis for ΔCT was performed (Figure 4). An area under the curve of 0.883 ± 0.37 (95%CI: 1.77 to 10.5, P = 0.000) was revealed. Here, 11.5 HU of ΔCT was the cut-off value used to differentiate esophageal cancer from post-inflammatory stricture. Thus, the criterion of ΔCT greater than 11.5 HU in the arterial phase was optimal for diagnosing cancer, with a sensitivity of 83.87%, a specificity of 84.85%, a positive predictive value of 83.87%, a negative predictive value of 84.85% and an accuracy of 84.38%.

In delayed phase the mean CT attenuation value of the background intact esophagus was 38.39 ± 7.88 HU (range, 22-56 HU) in all patients in groups A and B. In group A, the mean CT attenuation value in delayed phase was 71.66 ± 14.28 HU (range, 51-104 HU), and the mean ΔCT was 34.03 ± 15.94 HU. To distinguish

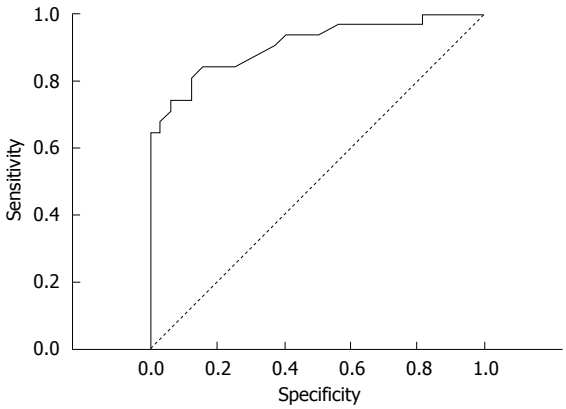

Figure 4 Receiver operating characteristic curve of ΔCT in arterial phase. A criterion of 11.5 HU in arterial phase showed optimal sensitivity (83.87%) and a specificity of 84.85% for the diagnosis of esophageal cancer.

the post-inflammatory strictures from the background normal esophageal walls, a ROC curve analysis for ΔCT was performed (Figure 5). An area under the curve of 0.845 ± 0.42 (95%CI: 2.5-23.3 P < 0.0001) was revealed, and 18.5 HU of ΔCT was the cut-off value. Thus, a ΔCT value more than 18.5 HU in delayed phase was optimal for determining the benign nature of stenosis, with a sensitivity of 93.75%, a specificity of 70.96%, a positive predictive value of 76.92%, a negative predictive value of 70.97%, and an accuracy of 82.54%. The mean attenuation of stenosis in group

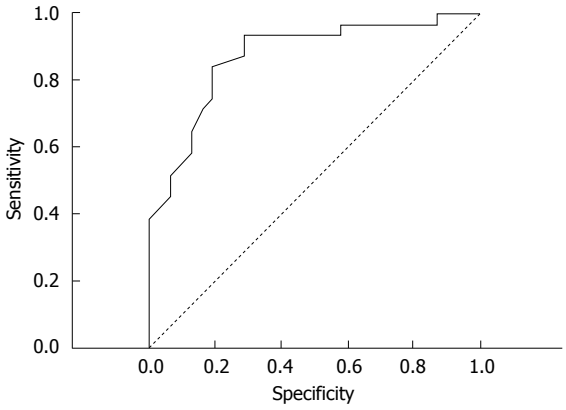

Figure 5 Receiver operating characteristic curve of △CT in delayed phase. A criterion of 18.5 HU showed optimal sensitivity (93.75%) and a specificity of 70.96% for the diagnosis of a benign esophageal stricture.

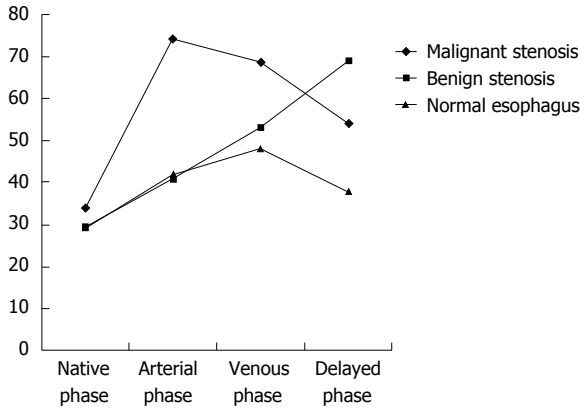

Figure 6 Graphs of mean attenuation. Graph of mean attenuation of normal esophageal wall, tumor and benign stenosis.

B showed a peak in the arterial phase, and the mean attenuation of stenosis in group A showed a peak in the delayed phase, whereas the attenuation of the intact esophageal wall tended to gradually enhance (Figure 6).

DISCUSSION

The normal thickness of the esophageal wall is 3-5 mm, depending on its extension[1,9]. Esophageal stenosis develops and clinically manifests as dysphagia when thickening of esophageal walls occurs and its lumen narrows to less than 13 mm. Many diseases can cause esophageal stricture formation, including peptic acid, autoimmune, infectious, caustic, congenital, iatrogenic, medication-induced, radiation-induced, malignant, and idiopathic disease processes. Double-contrast esophagography and endoscopy are the two major diagnostic modalities for evaluating the esophagus.

The most optimal staging diagnostic modalities for esophageal cancer currently combine EUS-guided fine needle aspiration (EUS-FNA) with either CT or PET scans[11-12]. EUS is important for tumor depth (T staging) and for regional lymph nodes (N staging) evaluation. Specificity of EUS for N staging increases by the use of EUS-FNA for lymph node cytology. But EUS examination is limited by tumors which obstruct the esophageal lumen. Up to 45% of tumors are non-traversable (most of these are T3-T4)[13]. In these cases dilation of esophageal lumen or the use of high frequency miniprobe ultrasound through an upper endoscope helps to overcome this limitation and is useful for staging of an obstructing tumor.

Although CT plays a significant role in the diagnosis and TNM staging of esophageal cancer[14-17], the CT findings obtained for post-inflammatory strictures are not well described in the radiology literature.

Esophageal wall thickening is a nonspecific response to various diseases[10]. The major objective in the CT assessment of unexplained esophageal wall thickening is to establish whether its cause is benign or malignant. Differential diagnosis of esophageal stenosis of various etiologies has been reported in the literature[6,18-20]. However, doubt may occasionally arise (in 10% of cases) in the accuracy of the diagnosis, despite the well-known CT-criteria used for the differential diagnosis of esophageal stenosis[4]. In this study we used MDCT with two-phase bolus contrast enhancement and hydro-CT to assess the capabilities of MDCT in the differential diagnosis of chronic inflammatory and tumor-induced changes in the esophagus. In our study, new features proved to be useful for determining esophageal stenosis in patients with post-inflammatory strictures.

We analysed the patients of in groups A and B with a variety of CT-findings that were repeated from patient to patient and identified the most significant features. Esophageal cancer is characterized by asymmetric wall thickening and the active accumulation of contrast medium during the arterial phase. Eccentric esophageal wall thickening, absence of the mucous membrane visualization in the stenosis, rupture of the mucous membrane at the upper boundary of stenosis, cup-shaped suprastenotic dilatation, tuberous boundaries of stenosis and luminal mass are most likely due to newly formed tumor tissue, which grows from the local area in the esophageal wall, destroys the mucosa and forms elevated borders of pathologically changed walls and luminal mass. However, cup-shaped suprastenotic dilatation was only found in 17 (54.8%) patients. These patients had T3 stage (9 patients), T4 stage (4 patients) or T1 and T2 stages (1 patient each) according to the TNM classification. Stage T1 and T2 esophageal cancer does not usually result in esophageal stenosis. This feature may be very helpful in diagnosing patients with moderate or significant stenosis. A pathologically high accumulation of the contrast medium in the arterial phase of bolus contrast enhancement occurred 10 s after a peak value in the aorta reached 120-150 HU. Our findings were consistent with those obtained in a study using triple-phase dynamic CT, where it was established that

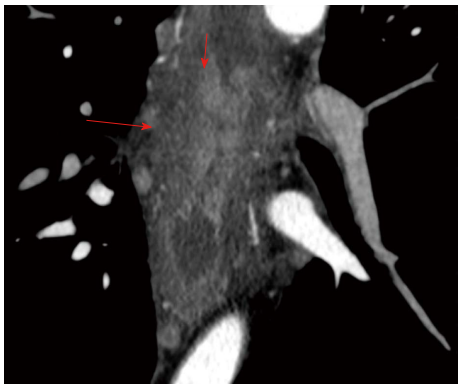

Figure 7 Atypical manifestation of corrosive esophageal stricture at computed tomography imaging. Arterial phase. Axial computed tomography (CT) scan of corrosive esophageal stricture. Development of mucosa granulations (short arrow), fibrotically changed submucosal and muscular layers and adventitia of esophageal walls (long arrow).

the maximum contrast enhancement of esophageal cancer occurred at the arterial phase of bolus contrast enhancement[19,21]. Clinically, the results of our study showed that the contrast-enhanced attenuation value within esophageal carcinoma was significantly higher than in the background normal esophageal wall in the arterial phase. Our findings suggested that the cut-off ΔCT of 11.5 HU in the arterial phase had high sensitivity, specificity, positive predictive value, negative predictive value and accuracy all of which were greater than 80%, in detecting esophageal cancer. Similar data were found in the research of Li *et al*[21]. Our findings also may be explained by the fact that esophageal carcinoma is typically hypervascular and that the formation of new arterial microvessels in tumors results in an increased enhanced attenuation value[22-24]. Therefore, the active accumulation of contrast medium in the arterial phase suggests the neoplastic origin of the stenosis, which was confirmed by our data.

Peptic stenosis is the most common cause of esophageal stenosis, and leads to the formation of 60%-70% of all benign strictures. Carrascosa *et al*[6] described peptic stenosis as short in length (less than 1 cm), with concentric esophageal wall thickening. Corrosive strictures can be variable in both length and number. We found these features to be insufficient, because in our research, 9 (29%) patients with esophageal cancer had concentric esophageal wall thickening and 3 (10%) patients with post-inflammatory strictures had eccentric esophageal wall thickening. Koehler *et al*[25] also noticed that malignant esophageal tumors may be manifested on CT because of concentric wall thickening. Gradual narrowing of the esophageal lumen occurs in chronic inflammatory stenoses and forms a conically shaped suprastenotic dilatation. Suprastenotic dilatation was found in most cases (87%) of benign stenosis with different thickness and length and is a very important feature that helps establish a correct diagnosis. The mucosa was often traceable in the arterial phase as a thin hyperintense line in the centre of hypodense thickened walls, caused by inflammation of the stricture (target sign) (Figure 4). Inside the stricture area, the mucosa can be frequently interrupted or may not be visualized when there is complete mucosal destruction. A "target sign" was found in the esophagus of 77% of the patients with post-inflammatory strictures. This sign is also typical of esophagitis[10]. We suggest that this feature is due to inflammation in the mucosa. A marked increase of attenuation in the post-inflammatory stricture in the delayed phase may be explained by the proliferation of fibrous tissue in its walls. It is well known that areas of delayed or prolonged enhancement in liver tumors at CT or magnetic resonance imaging correspond to fibrotic stroma at histopathologic examination[26-29]. In our research, fibrotic stroma was found in the histopathologic examination of all post-inflammatory strictures. The cut-off ΔCT of 18.5 HU in the delayed phase had high sensitivity, specificity, positive predictive value, negative predictive value and accuracy, all of which were greater than 70%, in the differential diagnosis of post-inflammatory stricture. Therefore, the CT attenuation value in delayed phase may be used as a criterion for discriminating benign stenosis from cancer.

The analysis of the post-inflammatory esophageal strictures revealed, an intraluminal component in two cases, which simulated neoplastic transformation in the wall. In one case, there was a soft-tissue component with a uniform structure that showed delayed accumulation of contrast medium, which was thus considered a fibrous tissue. In the other case, the soft-tissue component showed an active accumulation of contrast medium during the arterial phase and lymph nodes were enlarged up to 13 mm, which suggested a tumor (Figure 5). However, during analysis, the margin between the hyperintense component and the surrounding hypodense fibrous layers of the wall was clearly traced, which suggested a mucosal granulation and consequently an inflammatory origin for the stricture (Figure 7).

The CT differentiation of benign and malignant stenosis is important because the specific diagnosis influences the treatment, including the necessity of surgery and the technique, or surgical approach that should be used as well as the need for lymph node dissection.

In addition, the results of the study showed a significant enlargement of the regional lymph nodes (diameter more than 10 mm), which was typically associated with the neoplastic transformation of the esophageal wall.

Our study has some limitations, as represented by the low number of studied patients. Selection bias is another inevitable component in a retrospective study. Another limitation is that the measurement of CT enhancement is a semi-quantitative method for assessing vascularity, and is significantly affected by

the impact of patient cardiac output and central blood volume. To overcome this limitation, we measured the extent of CT enhancement within the stricture by subtracting the attenuation value of background intact esophageal walls from that of esophageal stenosis, which may help avoid the influence of cardiac output and central blood volume. The last limitation is that normal esophagus has thin walls and was thus more subject to partial volume averaging with adjacent paraesophageal fat tissue or air, which may have influenced the accuracy of the CT enhancement values measurements in the esophageal wall. To minimize this limitation, the measurements of CT enhancement were analysed on both thin-section and magnified images.

COMMENTS

Background

The correct diagnosis of benign or malignant stenosis in the esophagus is crucial for determining the appropriate treatment strategies. No detailed examination of the statistical significance of computed tomography (CT) findings of post-inflammatory esophageal strictures using two-phase dynamic multidetector CT (MDCT) has been published in the radiology literature. The authors' findings would be of high practical importance to radiologists.

Research frontiers

Corrosive and peptic strictures are characterized by morphologic and hemodynamic changes of the esophageal wall that occur due to chronic inflammation and result in fibrotic changes in the walls. To assess the typical changes in post-inflammatory strictures, the current research hotspot is the utilization of two-phase dynamic MDCT because both qualitative features, and perfusion changes can be quantitatively detected from contrast administration.

Innovations and breakthroughs

The characteristic CT appearances of esophageal stenosis of different etiologies have been described. Most previous studies have included unenhanced MDCT for evaluating the radiologic criteria of benign esophageal strictures. No detailed description of CT findings in post-inflammatory strictures has been published in the radiology literature. No study has analysed the value of MDCT with bolus contrast enhancement in the differential diagnosis of benign or malignant stenosis of esophagus. The authors reviewed data of two-phase dynamic MDCT in patients with benign and malignant stenosis and obtained qualitative and quantitative findings. They combined all such features and compared them with final histopathologic diagnoses. The most significant features in the differential diagnosis of post-inflammatory strictures from esophageal cancer were selected.

Applications

The features that were identified as significant for the CT evaluation of esophageal stenosis could be helpful in routine radiological practice and in treatment planning.

Terminology

Ultrathin esophagoscopy is impossible if the esophageal lumen is less than 5 mm thick. Additionally, radiologic and endoscopic diagnoses are not informative if total obstruction of the esophageal lumen occurs. Therefore, two-phase dynamic MDCT could be an imaging modality of choice in cases of severe stenosis, because it allows for the evaluation of esophageal walls below the area of stenosis.

Peer-review

This is an interesting research study that evaluates the utility of two-phase dynamic MDCT to differentiate post-inflammatory esophageal strictures from esophageal cancer. The study is well structured; the subject is an actual one and could be helpful in routine radiological practice. The described imaging findings show high diagnostic significance in differentiation benign strictures from esophageal cancer.

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ORIGINAL ARTICLE

Retrospective Study

Transcatheter arterial infusion for advanced hepatocellular carcinoma: Who are candidates?

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Eiichiro Suzuki, Tetsuhiro Chiba, Yoshihiko Ooka, Sadahisa Ogasawara, Akinobu Tawada, Tenyu Motoyama, Naoya Kanogawa, Tomoko Saito, Masaharu Yoshikawa, Osamu Yokosuka, Department of Gastroenterology and Nephrology, Graduate School of Medicine, Chiba University, Chiba 260-8670, Japan

Author contributions: Suzuki E, Ooka Y and Ogasawara S designed the research; Tawada A, Motoyama T and Kanogawa N performed the research and acquisition of data; Suzuki E, Ooka Y, Saito T and Yoshikawa M contributed to analysis and interpretation of data; Suzuki E and Chiba T performed the statistical analysis and wrote the manuscript; Yokosuka O supervised the study.

Institutional review board statement: The study was reviewed and approved by the Ethics Committee of Chiba University.

Informed consent statement: Written informed consent was obtained from all study participants prior to study enrollment.

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Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at techiba@faculty.chiba-u.jp. Participants gave informed consent for data sharing. No additional data are available.

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Abstract

AIM: To elucidate anticancer effects of transcatheter arterial infusion chemotherapy (TAI) in patients with hepatocellular carcinoma (HCC).

METHODS: Data from a total of 95 patients with HCC who received TAI were analyzed retrospectively. The efficacy of TAI was evaluated according to the Response Evaluation Criteria in Cancer of the Liver. Overall survival was calculated from the date of initial treatment to the date of death or last follow-up. Survival curves were calculated by the Kaplan-Meier method, and differences in survival were evaluated by the log rank test. Clinical variables that were identified as statistically different by a univariate analysis were included into the Cox proportional hazard regression model for multivariate analysis. A prognostic index based on the regression coefficients derived from variables identified by the multivariate analysis was constructed. Stratification of the patients was conducted using this prognostic index.

RESULTS: The patient group was comprised of 76 men and 19 women with an average age of 68 years (range: 37-82 years). Six patients (6.3%) showed

complete response and 18 patients (18.9%) showed partial response, for an overall response rate of 25.2%. The median overall survival was 27.6 mo, and the proportions of survivors at 1, 2, and 5 years were 67.4%, 54.0%, and 17.4%, respectively. Multivariate analysis demonstrated that no prior transcatheter arterial chemoembolization, lactate dehydrogenase < 230 IU/L, and performance status of 0 were the independent favorable prognostic factors. The development of a 0-3-point prognostic score index was based on the sum of these three prognostic factors. Subsequently, the patients were categorized into three groups: those with a good (prognostic index = 0-1; *n* = 54), intermediate (prognostic index = 2; *n* = 26), or poor (prognostic index = 3; *n* = 15) prognosis. The median survival times in these three groups were 41.0, 21.2, and 6.8 mo, respectively ($P < 0.01$).

CONCLUSION: Our simple prognostic index may be helpful for management of patients in determining treatment strategies for advanced HCC in the era of molecularly targeted therapy.

Key words: Hepatocellular carcinoma; Interventional radiology; Prognostic factor; Survival; Transcatheter arterial infusion

Core tip: Transcatheter arterial infusion chemotherapy is one of the therapeutic approaches for hepatocellular carcinoma. In this study, multivariate Cox regression analyses demonstrated that no prior transcatheter arterial chemoembolization, lactate dehydrogenase < 230 IU/L, and performance status of 0 were the independent favorable prognostic factors. The prognostic index based on a combination of these three prognostic factors successfully categorized the patients into three groups with good, intermediate, or poor prognoses. This index may assist in the prediction of response to transcatheter arterial infusion chemotherapy in patients with hepatocellular carcinoma.

Suzuki E, Chiba T, Ooka Y, Ogasawara S, Tawada A, Motoyama T, Kanogawa N, Saito T, Yoshikawa M, Yokosuka O. Transcatheter arterial infusion for advanced hepatocellular carcinoma: Who are candidates? *World J Gastroenterol* 2015; 21(29): 8888-8893 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i29/8888.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i29.8888

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common malignancy in the world[1,2]. Treatment options, such as resection, liver transplantation, and local ablative treatments, offer a chance of cure and improved life expectancy[3-7]. Transcatheter arterial chemoembolization (TACE) exhibits a marked antitumor effect in HCC, and meta-analyses have demonstrated that TACE improves the survival of patients with unresectable HCC and preserved hepatic function[8,9]. Recently, the multikinase inhibitor, sorafenib, has been also established as standard treatment for advanced HCC[10,11], but the survival rate remains unsatisfactory[12].

Transcatheter arterial infusion (TAI) is often used for the treatment of intermediate and advanced HCC, especially in Japan, because high concentration of the anticancer drugs can be delivered to tumors with less toxicity[13,14]. However, there is no evidence within phase Ⅲ trials that show a survival benefit. In a randomized controlled trial of TACE and TAI with zinostatin stimalamer and lipiodol, TAI yielded results comparable to those of TACE with respect to survival[15]. In addition, sorafenib has been used in TACE-refractory cases, but the survival advantage of this agent is modest. If TAI can be used as an alternative treatment option, the addition of embolization may not be necessary for HCC patients, and TAI may result in longer survival when compared with sorafenib. Although the identification of prognostic factors might help with appropriate patient selection for TAI, only a few reports have investigated these factors[16,17].

The present study was conducted to investigate the antitumor efficacy of the treatment, as well as to identify the prognostic factors in patients with advanced HCC receiving TAI. In addition, a prognostic index is proposed to assist with determining treatment strategies in patients with HCC.

MATERIALS AND METHODS

Patients

Between February 2000 and October 2010, 95 patients with advanced HCC were treated with TAI using various chemotherapy regimens at Chiba University Hospital, Japan. HCC was diagnosed on the basis of histologic examination or imaging studies such as distinctive findings on CT and/or angiography, with elevated serum levels of serum alpha-fetoprotein or des-γ-carboxy prothrombin. In principle, to assess the extent and size of the tumors before treatment, chest X-ray, ultrasonography, CT, and angiography of the abdomen were performed. Written informed consent was obtained from all the patients prior to the start of the treatment. This retrospective analysis was approved by the Ethics Committee of Chiba University.

Treatment procedure and assessment of efficacy

TAI was performed by selectively introducing a catheter into the artery feeding the tumor and injecting anticancer drugs in a similar fashion to TACE[18,19].

Table 1 Patient characteristics (n = 95)

Variables	Value
Sex, male/female	76/19
Age (yr): median (range)	68 (37-82)
ECOG-PS: 0/1/2	62/28/5
Etiology HBV/HCV/Others	72/12/11
Child-Pugh: A/B/C	49/42/4
Maximum tumor diameter (mm): median (range)	30.0 (10.0-160.0)
Tumor distribution: unilateral/bilateral	22/73
Portal vein tumor thrombosis: yes/no	19/76
AFP (ng/mL), median (range)	86.0 (1.9-5856.0)
DCP (mAU/mL), median (range)	117 (0-204030)
Previous treatment	
Surgical resection	4
PEI	25
RFA	8
TACE	34
None	24

ECOG-PS: Eastern Cooperative Oncology Group-performance status; HBV: Hepatitis B virus; HCV: Hepatitis C virus; AFP: Alpha-feto-protein; DCP: Des-γ-carboxy prothrombin; PEI: Percutaneous ethanol injection; RFA: Radiofrequency ablation; TACE: Transcatheter arterial chemoembolization.

Generally, the indication for TAI at our institution was as follows: (1) repeated TACE could not control the HCC progression; and (2) selective TACE could not be performed because of multilobar tumor expansion. Anticancer agents used for TAI were as follows: (1) epirubicin was selected as first-line treatment before cisplatin approval; (2) cisplatin-based regimens were used before miriplatin approval, but not in patients with chronic kidney disease; and (3) miriplatin was selected as first-line treatment after miriplatin approval. The dose of the drug and whether or not the port is implanted, were determined based on the tumor size and liver function. After the treatment, follow-up examinations, including CT, ultrasonography, tumor marker measurement, and serum biochemistry, were generally performed first at 1-3 mo after the treatment completion, and subsequently every 3-4 mo. The transcatheter arterial treatments were repeated when disease progression by imaging studies or clinical deterioration of the patient's general condition occurred.

Evaluation of the antitumor efficacy

The antitumor effect was assessed by CT or magnetic resonance imaging at 3 mo after treatment according to the Response Evaluation Criteria in Cancer of the Liver[20]. Lipiodol accumulation in the tumor was regarded as representing necrotic tissue. Complete response was defined as 100% size reduction or 100% necrosis of all tumors, and partial response was defined as > 50% reduction and/or necrosis in the sum of all measurable tumors. Progressive disease was defined as more than 25% tumor growth in the sum of all lesions and/or the appearance of any new lesions. Stable disease was considered as any effect that did not qualify for classification as complete or partial response or progressive disease.

Table 2 Chemotherapeutic regimens for hepatocellular carcinoma

Regimen	No. of patients
Cisplatin	35
Carboplatin	21
Epirubicin	13
SMANCS	12
Miriplatin	6
5-fluorouracil	5
Doxorubicin	2
Pirarubicin	1

SMANCS: Poly (styrene-co-maleic acid)-neocarzinostatin.

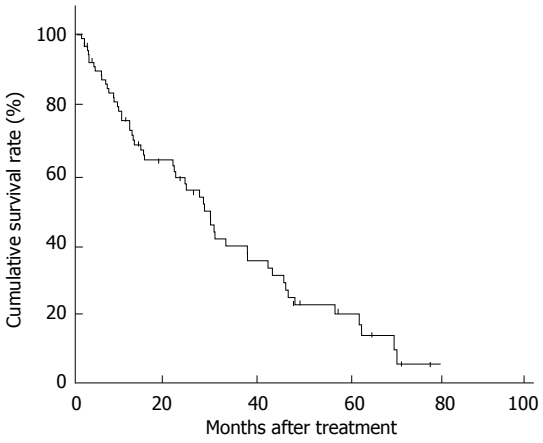

Figure 1 Overall survival curve for all patients with hepatocellular carcinoma treated by transcatheter arterial infusion chemotherapy. Tick marks indicate censored cases.

Statistical analysis

Variables were chosen based on previous investigations or our own clinical experience. Each of the variables was divided into two clinically meaningful subgroups. Overall survival was calculated from the date of initial treatment to the date of death or last follow-up. Survival curves were calculated by the Kaplan-Meier method. Univariate and multivariate analyses were calculated by Cox proportional hazards regression model. Only variables identified as statistically different by a univariate analysis were included in the multivariate analysis. A prognostic index based on the regression coefficients derived from all variables identified by the multivariate analysis was constructed. Stratification of the patients was based on this prognostic index. All P values shown in this report are of the two-tailed type. Differences at $P < 0.05$ were considered to be statistically significant.

RESULTS

Patient characteristics

The characteristics of all the 95 patients are shown in Table 1. The numbers of patients with Barcelona Clinic Liver Cancer stage A, B, C, and D were 6 (6.3%), 58 (61.1%), 27 (28.4%), and 4 (4.2%), respectively.

Table 3 Univariate and multivariate analyses of the prognostic factors of hepatocellular carcinoma patients receiving transcatheter arterial infusion chemotherapy

Variable	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Sex (male)	1.20 (0.60-2.37)	0.62		
Age (≥ 70 yr)	0.94 (0.59-1.70)	0.81		
ECOG-PS (1 or 2)	2.81 (1.58-5.02)	< 0.01	2.38 (1.29-4.39)	< 0.01
Platelet count (≥ 10.0 × 10^4/mm^3)	2.07 (1.22-3.53)	< 0.01		
AST (≥ 70 IU/L)	1.55 (0.92-2.61)	0.10		
ALT (≥ 53 IU/L)	1.23 (0.73-2.09)	0.44		
LDH (≥ 230 IU/L)	1.92 (1.14-3.22)	0.01	2.03 (1.19-3.45)	< 0.01
Total bilirubin (≥ 1.0 mg/dL)	1.88 (1.11-3.18)	0.02		
Albumin (≥ 3.0 g/dL)	0.80 (0.43-1.48)	0.47		
Prothrombin (≥ 70%)	0.48 (0.26-0.88)	0.02		
Maximum tumor diameter (≥ 30 mm)	1.27 (0.74-2.16)	0.39		
Tumor distribution (bilateral)	1.12 (0.62-2.02)	0.71		
Portal vein tumor thrombosis	1.78 (0.45-2.19)	0.14		
AFP (≥ 100 mg/ml)	1.25 (0.74-2.11)	0.40		
DCP (≥ 1000 mAU/mL)	1.20 (0.67-1.45)	0.33		
Prior TACE	2.38 (1.34-4.24)	< 0.01	2.34 (1.28-4.28)	< 0.01

HR: Hazard ratio; CI: Confidence interval; ECOG-PS: Eastern Cooperative Oncology Group-performance status; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; LDH: Lactate dehydrogenase; AFP: alpha-fetoprotein; DCP: Des-γ-carboxy prothrombin; TACE: Transcatheter arterial chemoembolization.

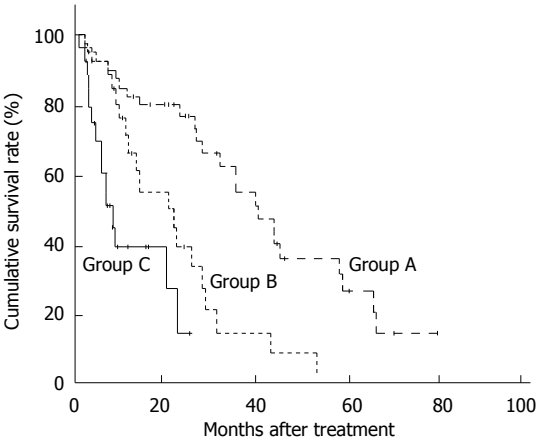

Figure 2 Survival curves for the three groups determined by a prognostic index. Group A: good prognosis; Group B: intermediate prognosis; Group C: poor prognosis. Tick marks indicate censored case.

Chemotherapy regimens of TAI are shown in Table 2. Thirty-five of 95 patients received hepatic arterial infusion chemotherapy using an implanted *port*-catheter system.

Treatment efficacy and survival

Six patients (6.3%) showed a complete response and 18 patients (18.9%) showed a partial response, for an overall response rate of 25.2%. The median survival time was 27.6 mo, and the proportions of survivors at 1, 2, and 5 years were 67.4%, 54.0%, and 17.4%, respectively (Figure 1).

Univariate and multivariate analyses

The results of univariate analysis and multivariate analysis using the Cox proportional hazard model are shown in Table 3. Among the factors, platelet ≥ 10.0 × 10^4/mm^3, total bilirubin < 1.0 mg/dL, lactate dehydrogenase (LDH) < 230 IU/L, performance status (PS) of 0, prothrombin time ≥ 70%, and no prior TACE, were significantly associated with longer survival time (all $P < 0.05$). In the multivariate analyses, only those variables identified as significant by the univariate analysis were examined. No prior TACE, serum LDH < 230 IU/L and PS of 0 were significantly associated with favorable survival.

Risk groups based on the regression model

To apply these findings clinically, a prognostic index was evaluated according to the regression coefficients derived from the three significant variables identified by multivariate analysis: prognostic index = score for prior TACE (0 for no, 1 for yes) + score for LDH (0 for < 230 IU/L, 1 for ≥ 230 IU/L) + score for PS (0 for PS 0, 1 for PS 1 or 2). The index values ranged from 0 to 3. The patients were then stratified into three groups according to the prognostic index, as follows: good prognosis group (prognostic index = 0-1; $n = 54$) (equivalent to patients with none or one of the three prognostic factors); intermediate prognosis group (prognostic index = 2; $n = 26$) (equivalent to patients with two of the three prognostic factors); poor prognosis group (prognostic index = 3; $n = 15$) (equivalent to patients with all of the three prognostic factors). The survival curves of these groups are shown in Figure 2. The median survival times in the good, intermediate, and poor prognosis groups were 41.0, 21.2, and 6.8 mo, respectively, with significant differences among the three groups ($P < 0.01$).

DISCUSSION

The aim of this study was to investigate the anticancer

activity of TAI and identify prognostic factors in patients with HCC receiving TAI. In this study, the median survival time and survival rates at two years in the current study were 27.6 mo and 54.0%, respectively. These results were comparable to those of TAI reported previously[16] and appeared to be favorable in comparison with those in conventional TACE performed approximately ten years ago[8,21]. However, recent studies[22-24] revealed that a new chemoembolization technique with drug eluting beads achieved longer survival than was observed in the present study.

Of importance, no prior TACE, LDH < 230 IU/L, and PS of 0 were found to be favorable prognostic factors by multivariate analysis. Many patients receiving TAI already have experienced TACE. One reason for unsatisfactory results is that HCC might acquire resistance to cytotoxic agents. LDH level correlates the tumor burden and the invasive potential of tumor. A high LDH level is associated with poor response to the therapy and recurrence of cancer in many malignancies[25,26]. PS is widely used as an assessment of the physical condition of the patient and is an important prognostic factor in patients with a variety of cancers[27,28]. For clinical application of these findings, we proposed a prognostic index based on the independent prognostic factors identified in this study. Patients are classified into three groups: those with good, intermediate, or poor prognoses. The median survival times in these three groups were 41.0, 21.2, and 6.8 mo, respectively. This index is easily calculated. Patients in the good prognosis group may obtain favorable response with TAI alone, with results comparable to those of TACE previously reported in Japan[29,30]. Even in TACE-refractory cases, TAI may be superior to sorafenib treatment if appropriate candidates are identified. In contrast, patients in the poor prognosis group are recommended for more aggressive treatments or best supportive care because of the extremely short median survival (6.8 mo).

The Barcelona Clinic Liver Cancer staging is widely used worldwide; in this staging system, TACE and sorafenib are the standard treatments for intermediate and advanced HCC, respectively[5]. Because of the lack of randomized controlled trials, TAI has not been recognized as an effective treatment. The combination of TAI with molecular targeted therapy may improve the survival for patients with advanced HCC.

This study has several limitations. First, the anticancer drugs used in TAI varied because no standard anticancer regimen has been established for TAI. Second, this study was retrospective and therefore has some inherent biases, such as selection criteria.

In conclusion, TAI exhibited antitumor effects and resulted in favorable survival in some patients with HCC. The prognostic factors identified and the proposed index based on these factors may be useful for predicting life expectancy, determining treatment strategies, and designing future clinical trials for advanced HCC in the era of molecularly targeted therapy.

COMMENTS

Background

Transcatheter arterial infusion chemotherapy (TAI) is one of the therapeutic approaches for hepatocellular carcinoma (HCC).

Research frontiers

In this study, the efficacy of TAI is re-evaluated. Additionally, the prognostic index was developed to determine who are candidates for TAI.

Innovations and breakthroughs

The prognostic index was developed based on the results of multivariate analysis, as follows: prognostic index = score for prior transcatheter arterial chemoembolization (0 for no, 1 for yes) + score for lactate dehydrogenase (0 for < 230 IU/L, 1 for ≥ 230 IU/L) + score for performance status (0 for a status of 0, and 1 for a status of 1 or 2).

Applications

The prognostic index described in the current study may be helpful for management of patients via determining treatment strategies for advanced HCC.

Terminology

TAI is an interventional radiologic treatment to deliver anticancer drugs to intrahepatic tumors.

Peer-review

The manuscript reported the efficacy of TAI on advanced HCC and investigated related prognostic factors by Cox regression analyses. The manuscript is well written and is significant to HCC management. As there is no other treatment control, such as TACE, related reports need to be cited and discussed in discussion section.

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ORIGINAL ARTICLE

Retrospective Study

Reversed portal flow: Clinical influence on the long-term outcomes in cirrhosis

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Author contributions: Maruyama H was responsible for the study concept and design; Kondo T, Maruyama H, Sekimoto T, Shimada T and Takahashi M collected the data; Kondo T and Maruyama H analyzed the data; Kondo T drafted the manuscript; and Maruyama H and Yokosuka O provided critical revisions of the manuscript for important intellectual content.

Institutional review board statement: This study was conducted in accordance with the Declaration of Helsinki, and was approved by the Ethics Committee of Chiba University Graduate School of Medicine.

Informed consent statement: Informed written consent for research use of medical records was obtained from all of the patients.

Conflict-of-interest statement: All of the authors declare that there are no potential conflicts of interest and that they have received no funding for this paper.

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Abstract

AIM: To elucidate the natural history and the longitudinal outcomes in cirrhotic patients with non-forward portal flow (NFPF).

METHODS: The present retrospective study consisted of 222 cirrhotic patients (120 males and 102 females; age, 61.7 ± 11.1 years). The portal hemodynamics were evaluated at baseline and during the observation period using both pulsed and color Doppler ultrasonography. The diameter (mm), flow direction, mean flow velocity (cm/s), and mean flow volume (mL/min) were assessed at the portal trunk, the splenic vein, the superior mesenteric vein, and the collateral vessels. The average values from 2 to 4 measurements were used for the data analysis. The portal flow direction was defined as follows: forward portal flow (FPF) for continuous hepatopetal flow; bidirectional flow for to-and-fro flow; and reversed flow for continuous hepatofugal flow. The bidirectional flow and the reversed flow were classified as NFPF in this study. The clinical findings and prognosis were compared between the patients with FPF and those with NFPF. The median follow-up period was 40.9 mo (range, 0.3-156.5 mo).

RESULTS: Twenty-four patients (10.8%) demonstrated NFPF, accompanied by lower albumin level, worse Child-Pugh scores, and model for end-stage liver disease scores. The portal hemodynamic features in the patients with NFPF were smaller diameter of the portal trunk;

presence of short gastric vein, splenorenal shunt, or inferior mesenteric vein; and advanced collateral vessels (diameter > 8.7 mm, flow velocity > 10.2 cm/s, and flow volume > 310 mL/min). The cumulative incidence rates of NFPF were 6.5% at 1 year, 14.5% at 3 years, and 23.1% at 5 years. The collateral vessels characterized by flow velocity > 9.5 cm/s and those located at the splenic hilum were significant predictive factors for developing NFPF. The cumulative survival rate was significantly lower in the patients with NFPF (72.2% at 1 year, 38.5% at 3 years, 38.5% at 5 years) than in those with forward portal flow (84.0% at 1 year, 67.8% at 3 years, 54.3% at 5 years, P = 0.0123) using the Child-Pugh B and C classifications.

CONCLUSION: NFPF has a significant negative effect on the prognosis of patients with worse liver function reserve, suggesting the need for careful management.

Key words: Non-forward portal flow; Reversed portal flow; Cirrhosis; Doppler ultrasound; Portal hemodynamics

Core tip: The influence of non-forward portal flow (NFPF) in cirrhosis has not been determined. The present study examined the effect of NFPF on the natural history of cirrhosis in 222 patients (median follow-up period 40.9 mo). The cumulative incidences of NFPF was as follows: 6.5% at 1 year, 14.5% at 3 years, and 23.1% at 5 years. The cumulative survival rate was significantly lower in patients with NFPF (72.2% at 1 year, 38.5% at 3 years, and 38.5% at 5 years) than in those with forward portal flow (84.0% at 1 year, 67.8% at 3 years, and 54.3% at 5 years, P = 0.0123) using Child-Pugh B and C classifications, suggesting the need for careful management of these patients.

Kondo T, Maruyama H, Sekimoto T, Shimada T, Takahashi M, Yokosuka O. Reversed portal flow: Clinical influence on the long-term outcomes in cirrhosis. *World J Gastroenterol* 2015; 21(29): 8894-8902 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i29/8894.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i29.8894

INTRODUCTION

The prevalence of chronic liver disease is increasing worldwide with an extensive range of etiologies[1-3]. Cirrhosis, which is the end stage of liver disease, continues one of the leading causes of death because of the increased risk for developing variceal bleeding, hepatic failure, and hepatocellular carcinoma (HCC)[3-7]. Managing cirrhosis should be based on the proper assessment of disease severity and the prediction of outcomes[8-10].

Portal hypertension, which represents the underlying pathophysiology of cirrhosis, is the basic mechanism responsible for several major complications. The impaired hemodynamics typified by the development of collateral vessels might explain the clinical presentations[3,11-13]. Doppler ultrasound (US), which is a frequently used diagnostic tool, offers real-time observation of hemodynamics with simple and reliable methodology[14-18]. It may also be the only method that can demonstrate the direction of blood flow in vessels under physiological conditions.

Non-forward portal flow (NFPF), *i.e.*, reversed or bidirectional flow in the portal venous system, is an abnormal but not rare condition in cirrhosis. The unique and impaired hemodynamics associated with NFPF are based on the anatomical features of the portal venous system and may represent a sign of advanced portal hypertension[19-22]. However, the influence of NFPF on the long-term clinical outcomes in patients with portal hypertension remains undetermined.

We designed the current study to compare the clinical manifestations and the long-term outcomes between patients with forward flow in the portal venous system and those with NFPF. The aim of the study was to elucidate the effect of portal flow direction on the long-term clinical outcomes of patients with cirrhosis.

MATERIALS AND METHODS

Study design

This retrospective study was based on the medical records in our department from June 2001 through November 2012. The information included the results of regular check-ups of patient's physical status, blood tests, and findings from endoscopic and Doppler US examinations. The study enrolled patients with cirrhosis who received Doppler US examinations for evaluating their portal hemodynamics. The diagnosis of cirrhosis was based on a combination of biochemical findings and US examination. The exclusion criteria were as follows: (1) patients with a malignant disease (including a history of HCC diagnosis based on radiological findings and/or histology[23]); (2) patients with less than 1 year of follow-up; (3) patients with more than a 1-year interval between Doppler US and endoscopic examinations; (4) patients receiving radiological or surgical treatment, such as stomach surgery, transjugular intrahepatic portosystemic shunt, or shunt embolization that may affect the flow direction in the portal vein; (5) patients receiving antiviral therapy during the study period; (6) patients using vasoactive drugs, such as β-blockers, because these agents are not approved as treatment for portal hypertension in Japan; and (7) patients with vascular abnormalities, such as an intrahepatic arterio-portal shunt diagnosed using Doppler US examination.

Hepatic encephalopathy (HE) was assessed using

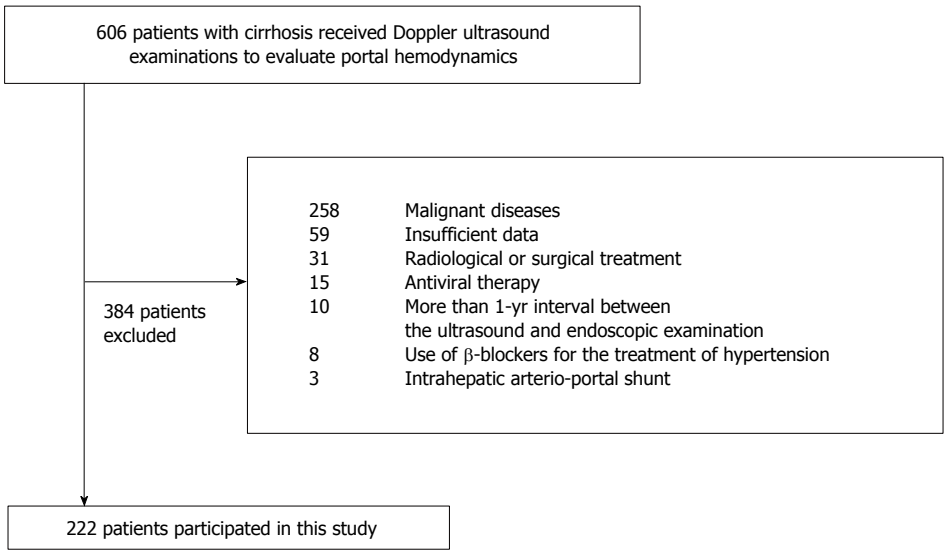

Figure 1 Patient enrollment. According to the inclusion and exclusion criteria, 222 participants were qualified eligible to participate in this study.

the West-Haven grading system[24], and grade Ⅱ or above was classified as overt HE. The degree of ascites was defined according to the following established guidelines[25]: mild for ascites that were only detectable by US examination, moderate for ascites that caused moderate symmetrical distention of the abdomen, and severe for ascites that caused marked abdominal distension. Moderate or severe ascites was classified as overt ascites. Portal vein thrombosis was defined as an echogenic structure that partially or completely occupied the lumen of the portal vein.

Decompensated cirrhosis was defined by the detection of at least one of the following presentations: variceal bleeding, overt ascites, overt HE, or jaundice (bilirubin level, > 3.0 mg/dL)[26,27]. The observation period was defined as the time between the initial US examination and the date of the last hospital visit, death, or liver transplantation.

Informed written consent for research use of medical records was obtained from all of the patients. This study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Chiba University Graduate School of Medicine.

Definition and treatment of varices

Gastroesophageal varices were assessed according to the general guidelines of the Japan Research Society for Portal Hypertension[28]. Variceal bleeding was defined by both of the following criteria: (1) presence of bleeding history, such as hematemesis or melena; and (2) endoscopic evidence of active bleeding or a fibrin clot on the varices.

The study applied endoscopic band ligation for the patients with active bleeding from esophageal varices followed by sclerotherapy. Regarding the primary prophylaxis in patients with medium/large grade varices, endoscopic sclerotherapy was the treatment of choice. However, repeated band ligation was performed in patients with poor liver function (Child-Pugh C classification) or advanced liver cancer. Meanwhile, in patients with active bleeding from gastric varices, cyanoacrylate injection therapy (Histoacryl, B. Braun, Melsungen AG, Germany) was performed to achieve hemostasis.

Characteristics of the study subjects

There were 606 consecutive patients with cirrhosis who underwent Doppler US examinations to evaluate portal hemodynamics during the study period. Because 384 patients were considered to be ineligible for this study according to the exclusion criteria (Figure 1), the study included a total of 222 patients: age range, 20-89 years; mean age ± SD, 61.7 ± 11.1 years; and 120 males, 102 females. The routine blood tests for chronic liver disease, including serum albumin, bilirubin, prothrombin time, and platelet count, were performed within 5.5 mo (median, 0.1 mo; range, 0-5.5 mo) before and after the Doppler US examinations. The follow-up US and physical examinations were performed at least once per year in all of the patients. The median observation period was 40.9 mo (range, 0.3-156.5 mo).

US examination

The US equipment used was an SSA-390A or an SSA-770A (Toshiba, Tokyo, Japan) and a 3.75-MHz convex probe. The examination was performed with the patients placed in the supine position after fasting for more than 4 h.

The portal hemodynamics were evaluated using both pulsed and color Doppler US[13,18]. The diameter (mm), flow direction, mean flow velocity (cm/s), and mean flow volume (mL/min) were assessed at the portal trunk, splenic vein (SV), superior mesenteric vein (SMV), and collateral vessels. The blood flow was

Table 1 Flow direction in the portal venous system *n* (%)

	Forward portal flow	Non-forward portal flow	
		Bidirectional flow	Reversed flow
Total	198 (89.2)	5 (2.3)	19 (8.6)
In portal trunk	-	2 (0.9)	4 (1.8)
In splenic vein	-	5 (2.3)	17 (7.7)
In superior mesenteric vein	-	2 (0.9)	0 (0)

Table 2 Portal hemodynamics using Doppler sonograms *n* (%)

	Forward portal flow	Non-forward portal flow	*P* value
Number of subjects	198	24	
Portal vein thrombosis (-/+)[1]	181/17	23/1	0.4538
Diameter of portal trunk (mm), mean ± SD (range)	11.9 ± 2.4 (4.9-22.7)	10.1 ± 2.0 (7.7-14.0)	0.0013
Collateral vessels (-/+)[1]	29/169	0/24	0.0443
Left gastric vein (hepatofugal)	137 (69.2)	9 (37.5)	0.0020
Short gastric vein (hepatofugal)	32 (16.2)	12 (50.0)	< 0.0001
Splenorenal shunt (hepatofugal)	16 (8.1)	6 (25.0)	0.0088
Paraumbilical vein (hepatofugal)	53 (26.8)	2 (8.3)	0.0482
Inferior mesenteric vein (hepatofugal)	9 (4.5)	4 (16.7)	0.0169
Characteristics of the largest collateral vessel			
Diameter > 8.7 (mm)[2]	26 (13.1)	17 (70.8)	< 0.0001
Flow velocity > 10.2 (cm/s)[2]	87 (43.9)	17 (70.8)	0.0126
Flow volume > 310 (mL/min)[2]	52 (26.3)	16 (66.7)	< 0.0001

[1]-: absence, +: presence; [2]Cut-off value was determined using the receiver operating characteristics analysis.

measured with a sampling width corresponding to the diameter of the vessel and an angle less than 60° between the US beam and the vessel. The average values from 2 to 4 measurements were used for the data analysis. The portal flow direction was defined as follows: forward portal flow (FPF) for continuous hepatopetal flow; bidirectional flow for to-and-fro flow; and reversed flow for continuous hepatofugal flow. The bidirectional flow and reversed flow were classified as NFPF in this study.

The US examinations were performed by Maruyama H or Takahashi M, who have more than 8 years of experience.

Statistical analysis

All of the data are expressed as the mean ± SD, median, or percentages. The comparisons were made between the patients with FPF and those with NFPF. The continuous variables were analyzed using Student's *t*-test or Mann-Whitney *U*-test, as appropriate. The categorical variables were analyzed using the χ^2 test. The best cut-off value was calculated based on the receiver operating characteristics (ROC) analysis. The cumulative survival rate was calculated using the Kaplan-Meier method, and multivariate analysis was performed using Cox regression analysis. A *P* value of less than 0.05 was considered to be statistically significant. Statistical analyses were performed using SAS version 9.4 software (SAS Institute, Cary, NC, United States).

RESULTS

Flow direction in the portal venous system

The patterns of the portal flow direction were forward in 198 patients (89.2%), bidirectional in 5 patients (2.3%), and reversed in 19 patients (8.6%). Therefore, NFPF was found in 24 patients (10.8%), portal trunk in 6 patients (2.7%), SV in 22 patients (9.9%), and SMV in 2 patients (0.9%) (Table 1).

The portal trunk diameter was smaller in the patients with NFPF than in those with FPF (*P* = 0.0013). The development of collateral vessels was closely associated with the portal flow direction (Table 2); the presence of the left gastric vein (LGV) (*P* = 0.0020) and the paraumbilical vein (*P* = 0.0482) was more frequent in the patients with FPF, whereas the presence of the short gastric vein (SGV) (*P* < 0.0001), the splenorenal shunt (SRS) (*P* = 0.0088), and the inferior mesenteric vein (*P* = 0.0169) was more frequent in those with NFPF.

The best cut-off values of diameter, flow velocity, and flow volume in the largest collateral vessel selected from each individual were calculated using the ROC analysis between the patients with FPF and those with NFPF, 8.7 mm for diameter (*P* < 0.0001), 10.2 cm/s for flow velocity (*P* = 0.0126), and 310 mL/min for flow volume (*P* < 0.0001) (Table 2).

Relationship between portal flow direction and clinical presentations at baseline

The presence of gastric varices was significantly more frequent in patients with NFPF than in those with FPF (*P* = 0.0093). The albumin level was lower in the patients with NFPF than in those with FPF (*P* = 0.0039). The Child-Pugh classification (*P* = 0.0015) and the model for end-stage liver disease (MELD) score (*P* = 0.0335) were worse in the patients with NFPF than in those with FPF (Table 3). The prevalence of decompensated cirrhosis did not differ between the patients with FPF and those with NFPF (*P* = 0.1074) (Table 3).

Development of decompensation

Among the patients with compensated cirrhosis at baseline, the development of decompensated cirrhosis during the study period was found in 40.2% (37/92) (16 overt ascites, 9 variceal bleeding, 8 jaundice, and 4 HE) of the patients with FPF, and 42.9% (3/7) (2 HE, and 1 jaundice) of the patients with NFPF, and the results did not differ significantly between the groups (*P* = 0.8909). However, regarding the causes of decompensation, the development of HE was observed to be significantly more frequent in the patients with NFPF (2/7) than in those with FPF (4/92) (*P* = 0.0096).

Table 3 Clinical findings in the subjects

	Forward portal flow	Non-forward portal flow	*P* value
Number of subjects	198	24	
Age (yr), mean ± SD (range)	61.9 ± 10.9 (35-89)	60.0 ± 12.6 (20-78)	0.4070
Sex (male/female)	110/88	10/14	0.1973
Etiology (Virus/Alcohol/NASH/PBC/AIH/others)	93/33/9/20/11/32	8/4/1/4/0/7	0.4003
Ascites (-/+/++)[1]	137/32/29	14/6/4	0.4949
Esophageal varices (-/+)[2]	73/125	10/14	0.6464
Gastric varices (-/+)[2]	155/43	13/11	0.0093
History of variceal bleeding (-/+)[2]	140/58	18/6	0.6610
History of variceal treatment (-/+)[2]	152/46	18/6	0.8469
Spleen (cm^2), mean ± SD (range)	28.9 ± 12.9 (10.3-99.6)	29.0 ± 16.4 (10.5-90.5)	0.9900
Hepatic encephalopathy (-/+)[3]	190/8	23/1	0.9764
Child-Pugh (A/B/C)	92/84/22	6/9/9	0.0015
Decompensated cirrhosis (-/+)[2]	92/106	7/17	0.1074
Model for end-stage liver disease score[4], mean ± SD (range)	11.0 ± 3.9 (6-24)	13.1 ± 4.8 (6-24)	0.0335
Blood test			
Bilirubin (mg/dL), mean ± SD (range)	1.8 ± 2.0 (0.3-16.9)	3.2 ± 3.3 (0.6-13.4)	0.0578
Albumin (g/dL), mean ± SD (range)	3.4 ± 0.6 (1.5-4.7)	3.1 ± 0.5 (1.8-3.9)	0.0039
Prothrombin time (%), mean ± SD (range)	69.0 ± 17.9 (29-133)	62.5 ± 21.4 (19-106)	0.1026
Platelet count (× 10^4/μL), mean ± SD (range)	9.5 ± 5.9 (1.7-43.9)	10.8 ± 6.0 (2.5-23.3)	0.2976

[1]Ascites -: none; +: mild; ++: moderate to severe: [2]-: absence; +: presence; [3]Hepatic encephalopathy -: grades 0-I; +: grades Ⅱ-Ⅳ (West-Haven grading system); [4]Forty-seven patients were excluded because prothrombin time-international normalized ratio was not measured in our hospital until 2004. AIH: Autoimmune hepatitis; NASH: Nonalcoholic steatohepatitis; PBC: Primary biliary cirrhosis.

Table 4 Cox regression analyses of the predictive factors for the development of non-forward portal flow

	Univariate hazard ratio (95%CI)	*P* value	Multivariate hazard ratio (95%CI)	*P* value
Diameter of portal trunk (< 11.2 mm)[1]	2.677 (1.269-5.648)	0.0097	-	-
Collateral vessels (flow velocity > 9.5 cm/s)[1]	2.818 (1.238-6.416)	0.0136	3.070 (1.276-7.389)	0.0123
Short gastric vein (hepatofugal)	3.039 (1.374-6.720)	0.0061	3.987 (1.736-9.158)	0.0011
Splenorenal shunt (hepatofugal)	3.485 (1.323-9.180)	0.0115	3.428 (1.143-10.278)	0.0279

[1]Cut-off value was determined using the receiver operating characteristics analysis.

Development of NFPF

Thirty-one patients developed NFPF from FPF during the study period, and the cumulative occurrence rates of NFPF were 6.5% at 1 year, 14.5% at 3 years, and 23.1% at 5 years. A univariate analysis showed that the diameters of the portal trunk < 11.2 mm (P = 0.0097) and the collateral vessels with flow velocity > 9.5 cm/s (P = 0.0136) were the best cut-off values using the ROC analysis and that the presence of SGV (P = 0.0061) and the presence of SRS (P = 0.0115) were significantly associated with the development of NFPF (Table 4). In the multivariate analysis, the collateral vessels with flow velocity > 9.5 cm/s (P = 0.0123), the presence of SGV (P = 0.0011), and the presence of SRS (P = 0.0279) were significant predictive factors for the development of NFPF (Table 4).

Among the patients with NFPF at baseline, the bidirectional flow changed to reversed flow in 1 patient, and the reversed flow changed to bidirectional flow in 3 patients during the study period.

Flow direction and survival

The cumulative overall survival rate was significantly worse in the patients with NFPF (79.2% at 1 year, 48.0% at 3 years, and 48.0% at 5 years) than those with FPF (90.9% at 1 year, 82.0% at 3 years, and 69.0% at 5 years; P = 0.0009) (Figure 2).

When stratified using Child-Pugh classification, there was no significant difference in the cumulative survival rate between the patients with FPF (98.9% at 1 year, 98.9% at 3 years, and 85.3% at 5 years) and those with NFPF (100% at 1 year, 75.0% at 3 years, and 75.0% at 5 years; P = 0.2314) using Child-Pugh A classification. However, the cumulative survival rate was significantly lower in the patients with NFPF (72.2% at 1 year, 38.5% at 3 years, and 38.5% at 5 years) than in those with FPF (84.0% at 1 year, 67.8% at 3 years, and 54.3% at 5 years; P = 0.0123) using Child-Pugh B and C classifications (Figure 3).

Twelve patients received liver transplantations, and 56 died during the study period: 39 died from hepatic failure, 8 died from HCC, 3 died from unknown cause, 2 died from variceal bleeding, 1 died from sepsis, 1 died from peritonitis of unknown origin, 1 died from a ruptured thoracic aortic aneurysm, and 1 died from heart failure.

DISCUSSION

The alternation in the blood flow direction is a unique

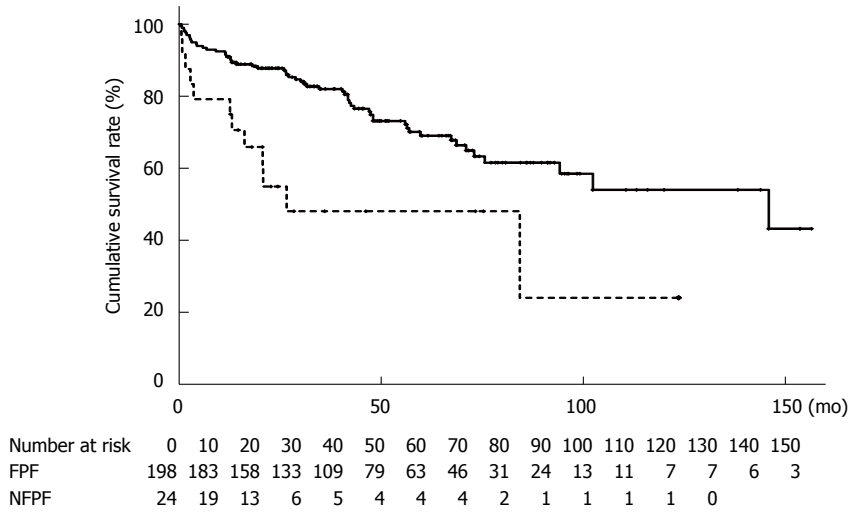

Figure 2 Cumulative survival rates between the patients with forward portal flow and those with non-forward portal flow. The cumulative survival rate was significantly worse in the patients with non-forward portal flow (79.2% at 1 yr, 48.0% at 3 yr, and 48.0% at 5 yr) than in those with forward portal flow (90.9% at 1 yr, 82.0% at 3 yrs, and 69.0% at 5 yr, $P = 0.0009$). Solid line: group with forward portal flow; dashed line: group with non-forward portal flow. FPF: Forward portal flow; NFPF: Non-forward portal flow.

phenomenon, that is observed in the portal venous system because of anatomical features and disease progression[19-21]. The present study focused initially on determining the frequency of this hemodynamic abnormality. NFPF was observed in 8.6% of the patients with reversed flow and 2.3% of the patients with bidirectional flow in the portal venous system in cirrhosis. This result may be comparable to the data in the literature, indicating that the overall incidence of reversed flow in the portal venous system in cirrhosis was 8.3% (3.1% in the portal vein, 3.1% in the SV, and 2.1% in the SMV)[20]. Other studies have reported frequency rates of 2.3% (3/132) in the portal trunk[29] and 9% in the intrahepatic portal vein[21]. However, presumably, the prevalence in chronic hepatitis is 1% in the SV, which is lower than the rate in cirrhosis[21].

Our study reported the cumulative incidence of NFPF, 6.5% at 1 year, 14.5% at 3 years, and 23.1% at 5 years, which have not been reported elsewhere. The presence of collateral vessels characterized by higher velocity (> 9.5 cm/s) or location at the splenic hilum was identified as significant hemodynamic factors associated with the future development of NFPF. In fact, there was no patient with NFPF who did not develop shunt vessels in our study. These data may suggest the influence of potentially advanced portal hypertension on the alternation of portal flow direction, and clearly encourage us to give these patients proper management.

Previous studies have shown a close relationship between flow direction in the portal venous system and liver function; hepatofugal flow was found to be more frequent in patients with Child-Pugh C (15.4%) and B (12.5%) classification than in those with Child-Pugh A (2.7%, $P < 0.02$) classification and was associated with a higher prevalence of HE (21% *vs* 7.2%; $P < 0.05$)[20]. Another study reported that reversed flow in the intrahepatic portal vein was significantly common in patients with Child-Pugh C (8/31, 25.8%) classification than in those with Child-Pugh A (0%)/B (5/104, 4.8%) classification[21]. One likely mechanism that could explain these results is the impaired nutritional metabolism with a poor hepatic clearance of toxic substance under the condition of NFPF[30]. Our study found that NFPF was closely associated with poor liver function, represented by serum albumin level, Child-Pugh classification, and MELD score. However, the portal flow direction was not a significant factor for the presence of liver decompensation at baseline in our study, which may be due to the definition of decompensation employed, *i.e.*, one of the major presentations is bleeding from the esophageal varices that is related to the development of LGV. In the present study, however, the prevalence of LGV was higher in the patients with FPF and there was no difference in the bleeding history between the patients with FPF and those with NFPF. Collectively, our data indicate that there is little, if any, relationship between NFPF and clinical decompensation.

A major clinical interest regarding NFPF in cirrhosis is its influence on the patient's prognosis. A previous study reported no significant difference in the survival rates between the patients with and without NFPF[20]. However, because the mean observation period in the study was only 13 mo (range, 12-18 mo), it may be difficult to draw a definite conclusion regarding the influence of NFPF on the long-term clinical course of patients with cirrhosis. The present study compared the long-term outcomes with a median observation time of 41 mo between the patients with FPF and those with NFPF. Although flow direction was not a significant survival factor in Child-Pugh A subjects, prognosis was significantly poorer in Child-Pugh B and C subjects with NFPF than in those with FPF. Thus, the influence

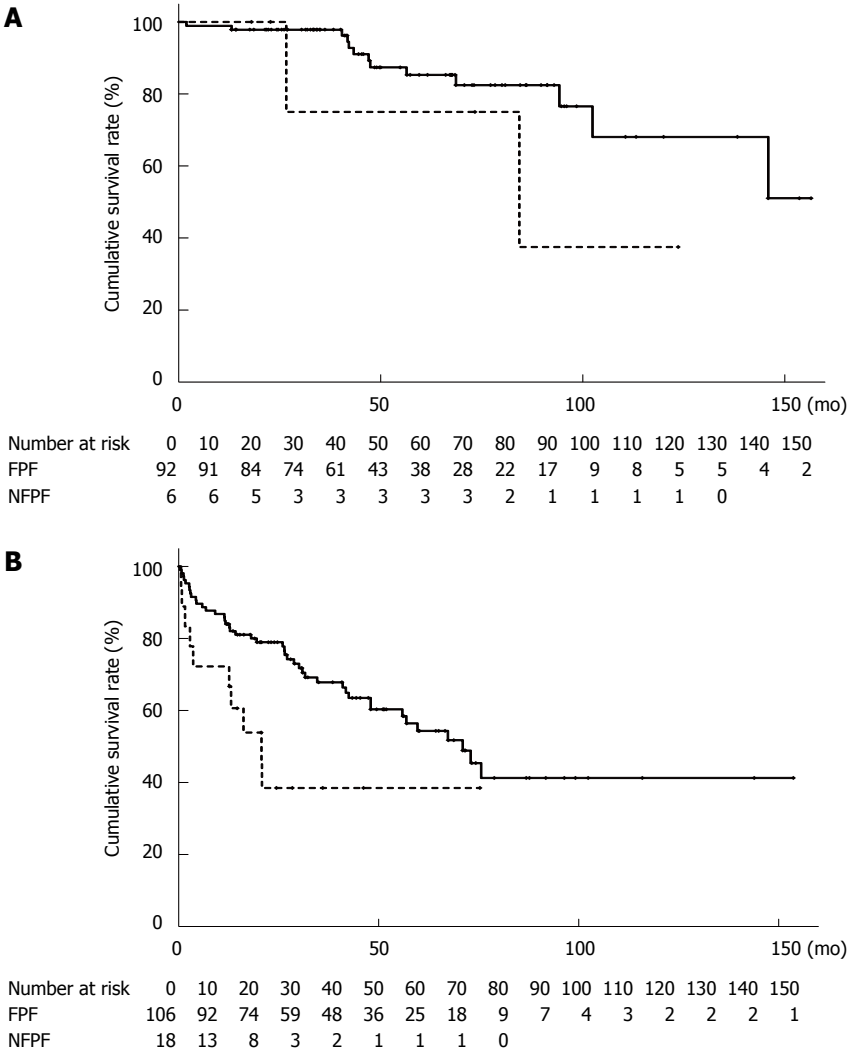

Figure 3 Cumulative survival rates between the patients with forward portal flow and those with non-forward portal flow. A: There was no difference in the cumulative survival rates between the patients with forward portal flow (98.9% at 1 yr, 98.9% at 3 yr, and 85.3% at 5 yr) and those with non-forward portal flow (100% at 1 yr, 75.0% at 3 yr, and 75.0% at 5 yr, $P = 0.2314$) using Child-Pugh A classification; B: The cumulative survival rate was significantly lower in the patients with non-forward portal flow (72.2% at 1 yr, 38.5% at 3 yr, and 38.5% at 5 yr) than in those with forward portal flow (84.0% at 1 yr, 67.8% at 3 yr, and 54.3% at 5 yr, $P = 0.0123$) using Child-Pugh B and C classifications. Solid line: group with forward portal flow; dashed line: group with non-forward portal flow. FPF: Forward portal flow; NFPF: Non-forward portal flow.

of portal flow on the patient's prognosis depends on the patient's liver function, which is an important point to consider in clinical practice.

Our study had several limitations. First, the data were obtained from the analysis under a retrospective setting. Therefore, the data must be validated in a prospective study. Second, our study lacks portal pressure data. The hemodynamics in patients with reversed flow direction should be assessed from the perspective of portal pressure in the future.

In conclusion, this longitudinal study has demonstrated the underlying hemodynamic mechanism, natural course and clinical influence of bidirectional or reversed portal flow in patients with cirrhosis. The presence of collateral vessels with high velocity and those at the splenic hilum are predictive features of the alternation of portal flow direction. Moreover, NFPF has a significant negative effect on the prognosis of patients with impaired liver function reserve. These observations highlight the usefulness of hemodynamic assessment using Doppler US, and suggest a likely direction for improving the clinical management of patients with cirrhosis.

COMMENTS

Background

Non-forward portal flow, *i.e.*, reversed or bidirectional flow in the portal venous system, is an abnormal but not rare condition in cirrhosis. However, the influence on the long-term clinical outcomes in patients with portal hypertension remains undetermined.

Research frontiers

Portal hemodynamic abnormality, which is the key pathophysiology of cirrhosis patients, may account for the various manifestations. It may determine the long-term outcomes, which are informative for the clinical management of these patients.

Innovations and breakthroughs

Non-forward portal flow is associated with the development of collateral vessels and poor liver function. Furthermore, non-forward portal flow has a significant negative effect on the prognosis of patients with worse liver function reserve.

Applications

Careful management may be needed in the clinical practice of cirrhotic patients with non-forward portal flow.

Terminology

Non-forward portal flow was defined for the bidirectional portal flow or reversed portal flow in this study.

Peer-review

This is a very solid study based on an interesting finding. The results of this study include important data.

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ORIGINAL ARTICLE

Retrospective Study

Clinical outcomes and ergonomics analysis of three laparoscopic techniques for Hirschsprung's disease

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Author contributions: Aubdoollah TH, Li K and Zhang X contributed equally to this work; Aubdoollah TH collected and analyzed the data, and drafted the manuscript; Li S and Yang L provided analytical oversight; Aubdoollah TH and Tang ST designed and supervised the study; Zhang X, Li K, Lei HY, Dolo PR, Xiang XC and Cao GQ revised the manuscript for important intellectual content; Zhang X, Lei HY and Dolo PR offered technical or material support; Wang GB and Tang ST provided administrative support; all authors have read and approved the final version to be published.

Institutional review board statement: The Ethics Committee of Huazhong University of Science and Technology has reviewed and approved this study.

Informed consent statement: Before the consent form was signed by the parent, they were fully informed about the surgical procedure with the associated risks, additional trocar, and possibility for conversion to the open technique.

Conflict-of-interest statement: This study was supported by the Public Welfare Research and special funds were received from the National Health and Family Planning of China (No. 201402007).

Data sharing statement: The technical appendix, statistical code, and dataset are available from the corresponding author at tshaotao83@126.com. All the participants gave informed consent for data sharing when they enrolled in this study. No additional data are available.

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Abstract

AIM: To report the clinical outcomes and ergonomics analysis of three laparoscopic approaches in the management of Hirschsprung's disease (HD).

METHODS: There were 90 pediatric patients (63 boys, 27 girls; mean age: 3.6 ± 2.7 mo; range: 1.0-90.2 mo) who underwent laparoscopic endorectal pull-through Soave procedures for short- and long-segment HD in our hospital. Three laparoscopic approaches were used: conventional laparoscopic pull-through (CLP) in 30 patients between 2009 and 2013, single-incision laparoscopic pull-through (SILP) in 28 patients between 2010 and 2013, and hybrid single-incision laparoscopic pull-through (H-SILP) in 32 patients between 2011 and 2013. We applied the hybrid version of the single-

incision approach in 2011 to preserve the cosmetic advantage of SILP and the ergonomic advantage of CLP. We retrospectively analyzed the clinical data, cosmetic results, and ergonomics of these three approaches to have a better understanding of the selection of one approach over another.

RESULTS: The CLP, SILP, and H-SILP groups were similar in regard to age, sex, transition zone, blood loss, hospital stay, and intraoperative complications. Early and late postoperative results were not different, with equal daily defecation frequency and postoperative complications. No conversion to open technique was needed and none of the patients had recurrent constipation. With proper training, the ergonomics challenges were overcome and similar operative times were registered for the general operative time in the patients < 1 year of age and the short-segment HD patients. However, significantly shorter operative times were registered compared to SILP for patients > 1 year of age (CLP and H-SILP: 120 ± 15 min and 119 ± 12 min, respectively, *vs* 140 ± 7 min; $P < 0.05$) and for long-segment HD patients (152 ± 3.5 min and 154 ± 3.6 min, respectively, *vs* 176 ± 2.3 min; $P < 0.05$). The best cosmetic result was registered with the SILP (scarless), followed by the H-SILP (near scarless appearance) and the CLP (visible scars) procedures.

CONCLUSION: Based on the results, we believed that the laparoscopic approach should be selected according to the age, transition zone, and desired cosmetic result.

Key words: Age; Cosmetic; Ergonomic; Hirschsprung's disease; Laparoscopic pull-through

Core tip: This manuscript describes a comparison of the (1) hybrid single-incision laparoscopic endorectal pull-through, (2) conventional laparoscopic endorectal pull-through, and (3) single-incision laparoscopic endorectal pull-through in selected Hirschsprung's disease patients. The cosmetic advantage was not the only concern, but also the age and transition zone-relating aspects of these three approaches. The clinical outcomes and ergonomics analysis are reported in order to better understand the choice of one approach over the other with regard to the patient's age, transitional zone, and desired cosmetic result.

Aubdoollah TH, Li K, Zhang X, Li S, Yang L, Lei HY, Dolo PR, Xiang XC, Cao GQ, Wang GB, Tang ST. Clinical outcomes and ergonomics analysis of three laparoscopic techniques for Hirschsprung's disease. *World J Gastroenterol* 2015; 21(29): 8903-8911 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i29/8903.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i29.8903

INTRODUCTION

Hirschsprung's disease (HD) is a well-known disease among pediatric surgeons; it is defined as a congenital abnormality of the enteric nervous system with the absence of ganglion cells in the distal parts of the colon resulting in a functional obstruction[1].

The laparoscopic technique has improved the surgical treatment of HD and has inspired surgeons to undertake more complex laparoscopic procedures to (1) promote early resumption of gastrointestinal function; (2) decrease complications; and (3) achieve better cosmetic results. Georgeson *et al*[2] described the conventional laparoscopic pull-through (CLP) procedure in 1995, and the single-incision laparoscopic pull-through (SILP) procedure was reported by Muensterer *et al*[3] in 2010. Since 1999, we have adopted the CLP procedure to treat various types of HD patients. In 2010, we started to perform SILP for cosmetic reasons, however, this procedure was challenging. In 2011, after accumulating a large amount of experience in CLP[4-6] and SILP[7], we applied the hybrid single-incision laparoscopic pull-through (H-SILP)[8], a combination of both procedures that preserves the cosmetic advantage of SILP and the ergonomic advantage of CLP.

This report describes the main three laparoscopic approaches that we have been using to treat HD with the same endorectal pull-through modified Boley-Soave procedure. The clinical outcomes and ergonomics analysis of the results are reported in order to better understand the choice of one approach over the other with regard to the patient's age, transitional zone, and desired cosmetic result.

MATERIALS AND METHODS

Patients

The clinical data of 90 cases (63 boys, 27 girls; mean age: 3.6 ± 2.7 mo, range: 1.0-90.2 mo) who underwent laparoscopic pull-through modified Soave procedures for short- and long-segment HD between 2009 and 2013 were retrospectively compared. Three laparoscopic approaches were used, including CLP in 30 patients between 2009 and 2013, SILP in 28 patients between 2010 and 2013, and H-SILP in 32 patients between 2011 and 2013.

The diagnosis was established in all the patients by rectal biopsy and anorectal manometry before the surgery[9,10]. Contrast barium enema[11] was performed to estimate the extent of the disease. The intraoperative frozen section biopsies and postoperative pathology reports confirmed the absence of ganglion cells and the transitional zone. Hematoxylin and eosin staining and calretinin and microtubule-associated protein-2 immunostaining were used to determine the presence or absence of the ganglion cells[12].

All these 90 patients underwent the same coloanal

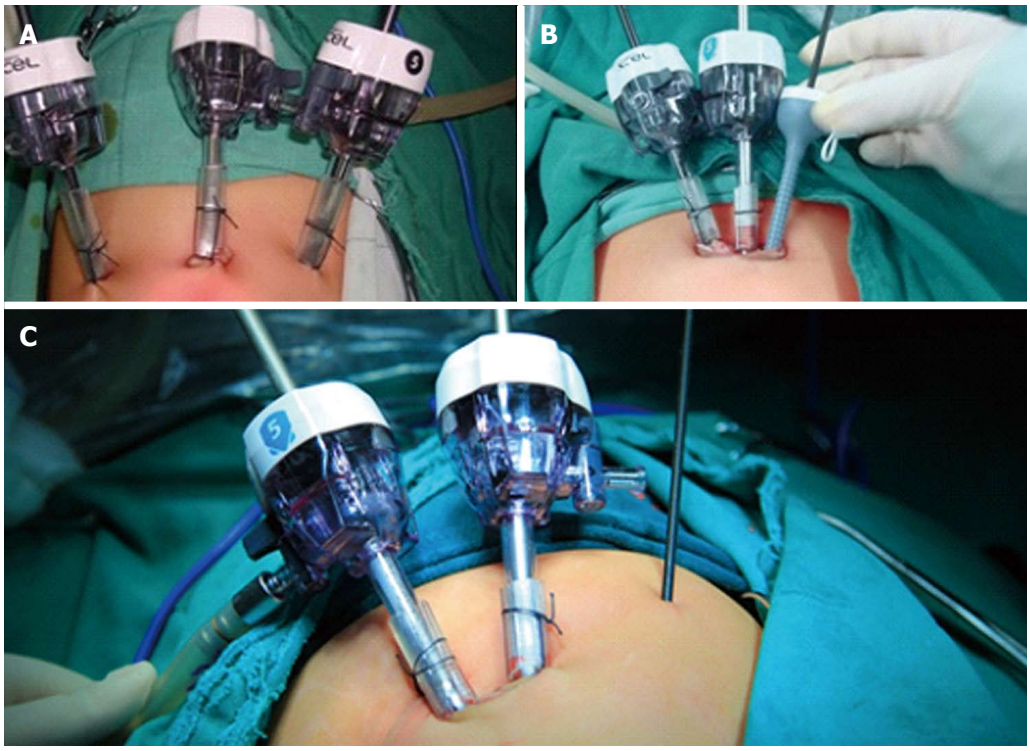

Figure 1 Positioning of trocars and instruments. A: Conventional laparoscopic pull-through; B: Single-incision laparoscopic pull-through; and C: Hybrid single-incision laparoscopic pull-through procedure.

anastomosis. The transitional zone was located in the rectosigmoid (80 cases) and distal descending colon (10 cases). Patients who (1) required total or subtotal colectomy; (2) were treated by other procedures than the modified Boley-Soave procedure; and (3) were lost to follow-up or were previously operated in other hospitals were not included in this study. We also excluded three patients from the SILP group with long-segment HD (aged 22, 34, and 49 mo), because an additional working port was added to the right lower abdomen in order to retract the huge elongated colon for better exposure and to facilitate the dissection. Otherwise, it would have been very difficult to expose the vascular arcades of the descending colon and mobilize the splenic flexure. The operative time for the patients < 1 year (n = 63; infant) and > 1 year (n = 27; toddler, preschooler, and school-age) and the transitional zone of the three groups were compared to analyze the ergonomic impact or difficulties of the different laparoscopic approaches related to the age and transitional zone. None of the patients had a previous colostomy. Preoperatively, daily colon irrigations (mechanical bowel preparation) were performed for 2-7 d. Usually, intravenous antibiotics were started 1-2 d prior to operation and stopped 3 d after operation. Patients were reviewed on a monthly basis for 6 mo postoperatively, and then every 3-6 mo with mean follow-up 36 ± 10 mo (range: 17-53 mo).

Surgical procedure

All the procedures were performed under endotracheal general anesthesia. Cleaning and draping was performed for intraoperative change of position from the laparoscopic to transanal phase. Usually, the surgeon would position himself at the head or on the right of the table, facing the monitor, and the table was adjusted with the patient lying in a 30° head-down position.

In all the three groups of patients, the surgery was performed in two phases: (1) the laparoscopic phase; and (2) the transanal phase.

Laparoscopic phase: The CLP, SILP, and H-SILP procedures differ from each other with regard to the positioning of the trocars and instruments (Figure 1), but the objective remains the same. Usually, one assistant surgeon was needed for handling the 30° straight laparoscopic camera. No additional trocar was used.

In CLP, the first 5.0 mm trocar was introduced in the umbilicus *via* an umbilical skin incision as a laparoscope port. Under vision, after pneumoperitoneum was stabilized, two additional 5.0 mm trocars were introduced in the abdomen at the respective position: one on the left side and one on the right side (Figure 1A).

In SILP, the first 5.0 mm trocar was introduced in the umbilicus centrally *via* a single vertical umbilical skin incision as a laparoscope port, and pneumoperitoneum was stabilized. Lateral to the laparoscopic port, two additional trocars were introduced into the abdominal cavity as operator ports after the skin was stretched horizontally (one 5.0 mm trocar introduced on the

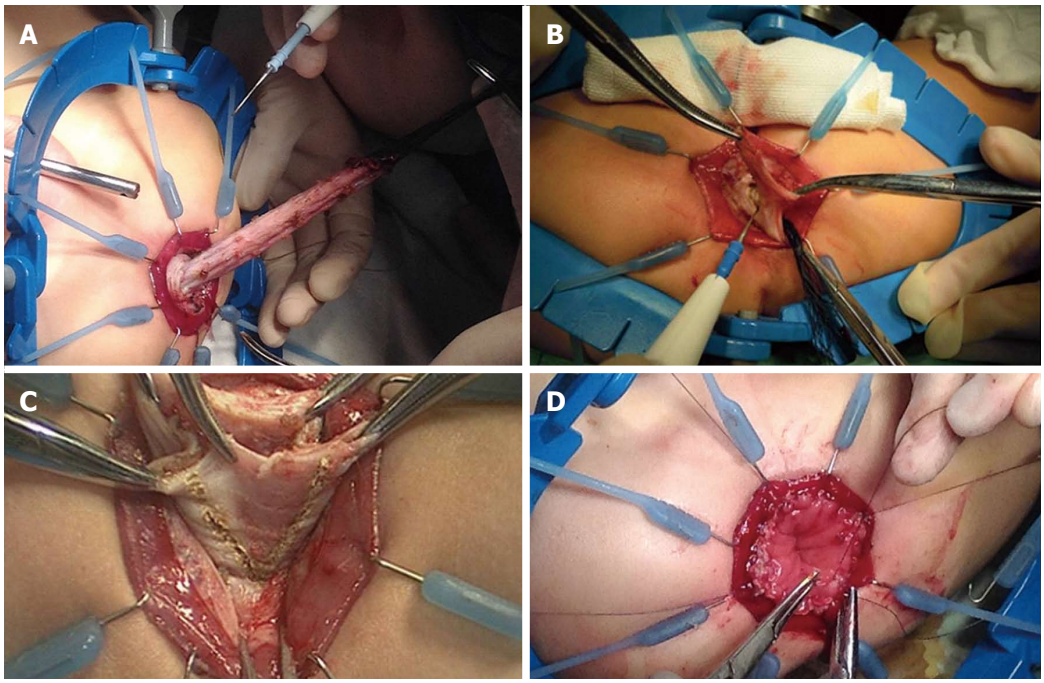

Figure 2 Resection of the necessary amount of the mobilized colon and coloanal anastomosis with the short cuff. A: Rectal submucosa dissection with a long cuff; B: Cuff shortening; C: Partial resection of the muscular cuff with a "V" shape of the posterior wall; D: Coloanal anastomosis after the endorectal pull-through and resection of the necessary amount of colon.

right for an ultrasonic scalpel and one 3.0 mm trocar introduced on the left for a grasping forceps) (Figure 1B).

In H-SILP, two 5.0 mm trocars were introduced in the abdominal cavity *via* a vertical incision at the umbilicus. After pneumoperitoneum was stabilized, the left port was used as the laparoscope port and the right port was used as the working port for the ultrasonic scalpel or grasping forceps. A trocarless 3.0 mm grasping forceps was punctured in the abdomen *via* a stab incision, 10 cm to the left side of the umbilicus (Figure 1C).

After the insertion of the laparoscopic instruments, an overall view was obtained and the transition zone was located. One or two seromuscular biopsies were obtained for frozen section histology to identify the ganglionic bowel and to decide the dissecting level of the mesentery. Using the ultrasonic scalpel, a window was made in the sigmoid mesentery and the rectosigmoid colon was mobilized 5 cm above the transitional zone by dissecting the mesentery and relative vessels; however, the marginal arcade was preserved. The colonic pedicle was freed with sufficient length that it could be pulled through without tension or overstretching. The dissection was stopped at the rectal peritoneal reflection. After the laparoscopic dissection, the ports were left *in situ*, and we changed our position for the transanal phase. For long-segment HD patients, mobilization of the colon was performed to a higher level.

Transanal phase: All patients underwent the same modified Boley-Soave's endorectal pull-through procedure[4-13]. This modified procedure consisted of: (1) lesser dissection in the pelvic cavity by the harmonic scalpel; (2) development of a long muscular rectal cuff for > 5 cm up to the peritoneal reflection by dissecting the submucosa layer of the rectum using electrocautery, which was then shortened to 2-3 cm; (3) partial resection of the rectal muscular cuff in a "V" shape at the posterior wall; and (4) resection of the necessary amount of the mobilized colon and coloanal anastomosis with the short cuff (Figure 2).

Once the anastomosis was completed, a rectal tube was inserted and laparoscopy was performed again to check for orientation of the pull-through bowel. Chitogel (15 mL of medical chitosan and physiologic-balanced solution) was injected at the dissection site *via* one of the ports to prevent peritoneal adhesion[14]. No abdominal drain was inserted. The port site at the umbilicus was stitched by 2-0 vicryl. The skin of the umbilicus and the other incisions were closed with skin glue. The postoperative appearance of the umbilicus and abdominal wound in the three groups were compared (Figure 3).

Postoperatively, patients were kept on intravenous total parenteral nutrition[15] and nasogastric decompression for 12-24 h. Intravenous antibiotics were given for 72 h and the urinary catheter was removed after 72 h. Patients were fed orally when bowel sounds returned. The parents were instructed on how to care for the wound and with regard to the toileting of the patients. The patients were discharged when they were clinically stable. Each patient was reviewed 2 wk after the operation, when a digital rectal examination

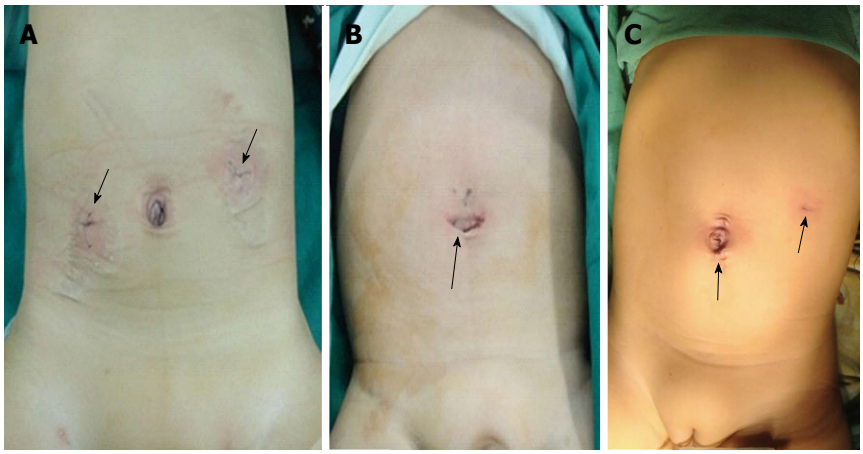

Figure 3 Postoperative appearance of umbilicus and abdominal wound. A: Conventional laparoscopic pull-through; B: Single-incision laparoscopic pull-through; and C: Hybrid single-incision laparoscopic pull-through procedure.

Table 1 General characteristics, operative data, and complications *n* (%)

Characteristic	CLP (*n* = 30)	SILP (*n* = 28)	H-SILP (*n* = 32)	*P* value
Median age (mo) (range)	3.8 ± 2.6 (1.0-90.2)	3.4 ± 2.1 (1.0-78.8)	3.6 ± 2.1 (1.0-78.6)	> 0.05
Sex, male	21 (70.0)	18 (64.3)	24 (75.0)	> 0.05
General operative time (min)	115 ± 22 (75-156)	118 ± 22 (90-178)	115 ± 24 (75-158)	> 0.05
Conversion to open surgery	0	0	0	> 0.05
Estimated blood loss (mL)	5.0 ± 1.0	6.0 ± 1.5	4.5 ± 1.0	> 0.05
Hospital stays (d)	7.0 ± 1.5	7.0 ± 1.0	7.0 ± 1.0	> 0.05
Intraoperative complications	0	0	0	> 0.05
Time of passage of flatus (h)	22.0 ± 5.0	21.5 ± 4.5	21.5 ± 4.0	> 0.05
Early postoperative complications	11 (36.7)	8 (33.3)	8 (31.3)	> 0.05
Perianal excoriation	10 (33.3)	7 (25.0)	9 (28.1)	
Anastomotic leak	1 (3.3)	0	0	
Enterocolitis	0	1 (3.6)	1 (3.1)	
Defecation frequency, times per day				
1 wk postoperatively	5 ± 4	6 ± 4	5 ± 4	> 0.05
1 mo postoperatively	4 ± 2	4 ± 3	4 ± 2	> 0.05
3 mo postoperatively	2 ± 1	2 ± 1	2 ± 1	> 0.05
Recurrent constipation	0	0	0	> 0.05

CLP: Conventional laparoscopic pull-through; SILP: Single-incision laparoscopic pull-through; H-SILP: Hybrid single-incision laparoscopic pull-through.

was performed and anal dilatation[16,17] was taught to the parents, so that they could perform it at home with Hegar dilators once daily for 3-6 mo, until the dilatation process became easy and painless with the recommended dilator size.

Statistical analysis

The SPSS 13.0 software package (SPSS Inc., Chicago, IL, United States) was used for data analysis. The transition zone, age at surgery, operative time, estimated blood loss, intraoperative complications, conversion to open surgery, time of flatus passage, postoperative hospital stay, and defecation frequency were compared using analyses of variance. The sex and postoperative complications were compared using the χ^2 test. The Student's *t* test was used to compare the mean scar score between the CLP and the H-SILP groups. All the results are described as mean ± SD or as percentage. A $P < 0.05$ was considered as significant. The statistical methods of this study were reviewed by Prof. Ping Yin, PhD (Department of Epidemiology and Biostatistics School of Public Health Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China.

RESULTS

General characteristics, operative data, and complications

The CLP, SILP, and H-SILP groups were similar in regard to age, sex, transition zone, blood loss, general operative time, hospital stay, and intraoperative complications (Table 1). Early and late postoperative results were not different, including equal daily defecation frequency and postoperative complications. There was no conversion to open technique. Postoperative enterocolitis occurred in two patients, one from the SILP (1/28; 3.6%) and the other from then H-SILP (1/32; 3.3%) group; both were treated by intravenous fluid, antibiotic, parenteral nutrition, and enemas during re-hospitalization.

Table 2 Operative time among the three procedure groups relative to the age and transitional zone

Group	CLP (n = 30)	SILP (n = 28)	H-SILP (n = 32)
Patients < 1 yr of age, n	21	20	22
Operative time (min)	113 ± 23	109 ± 8	113 ± 12
Patients > 1 yr of age, n	9	8	10
Operative time	120 ± 15[a]	140 ± 7	119 ± 12[a]
Rectosigmoid colon, n	27	26	27
Operative time (min)	111 ± 20	114 ± 17	109 ± 20
Descending colon, n	3	2	5
Operative time (min)	152 ± 4[a]	176 ± 2	154 ± 4[a]

[a]P < 0.05 *vs* SILP. CLP: Conventional laparoscopic pull-through; SILP: Single-incision laparoscopic pull-through; H-SILP: Hybrid single-incision laparoscopic pull-through.

Table 3 Cosmetic assessment

Assessment	CLP (n = 30)	SILP (n = 28)	H-SILP (n = 32)	P value
Number of visible scars on the abdomen	2	0	1	-
Scar appearance on the abdomen	Visible	Scarless	Near scarless	-
MSS score of visible scar on the abdomen	10.00 ± 0.72 (good)	-	5.00 ± 0.72 (excellent)	< 0.05
Appearance at the umbilicus	Normal	Normal	Normal	-

CLP: Conventional laparoscopic pull-through; SILP: Single-incision laparoscopic pull-through; H-SILP: Hybrid single-incision laparoscopic pull-through; MSS: Manchester scar scale.

Anastomotic leak occurred in one case (1/30; 33.3%) from the CLP group and was treated conservatively with a rectal decompressing tube, intravenous antibiotics, and total parenteral nutrition to allow the leak to heal by itself. Perianal excoriation occurred in 26/90 (28.8%) patients (n = 7 SILP, n = 10 CLP, and n = 9 H-SILP), and was treated by keeping the perianal are clean and dry and with application of stomahesive. All the patients achieved normal defecation without incontinence or recurrent constipation with a mean of 3.0 mo to obtain normal defecation frequency (1-2 times/d).

Operative time among the three groups relative to the age and transitional zone

The operative times did not differ among the patients < 1 year of age, but were significantly shorter for CLP and H-SILP compared to SILP for patients > 1 year (P < 0.05) (Table 2). Similarly, the operative times among short-segment (rectosigmoid) HD patients did not differ, but were significantly shorter for CLP and H-SILP compared to SILP in long-segment (descending colon) HD patients (P < 0.05).

Cosmetic assessment

The wounds were healthy at hospital discharge and healed by 3 wk postoperatively. The cosmetic assessment was conducted 12 mo postoperatively (Table 3). The assessment of the abdomen revealed two visible scars in the CLP patients (Figure 4A), one barely remarkable/near scarless scar in an H-SILP (Figure 4C) patient and a scarless abdomen in the SILP patients (Figure 4B). The scar at the umbilicus was unremarkable, as it was embedded inside and the umbilicus appeared normal in all the patients. The Manchester Scar Scale[18,19] was used to assess the scar score only for the CLP and H-SILP patients, showing a significant difference between the two groups (P < 0.05) (Table 4). None of the patients suffered from wound infection or complications such as keloids or hypertrophied scars.

Table 4 Manchester scar scale: 5 (best) to 18 (worse)

Category	Visual analog scale descriptor	Poor
Color	Perfect	1
	Slight mismatch	2
	Obvious mismatch	3
	Gross mismatch	4
Matte *vs* shiny	Matte	1
	Shiny	2
Contour	Flush with surrounding skin	1
	Slightly proud/indented	2
	Hypertrophic	3
	Keloid	4
Distortion	None	1
	Mild	2
	Moderate	3
	Severe	4
Texture	Normal	1
	Just palpable	2
	Firm	3
	Hard	4

DISCUSSION

The advancements in minimally invasive techniques have allowed HD to be treated quickly and safely with well-known benefits such as minimal surgical trauma, short operative time, and less postoperative pain[20], which lead to fast rehabilitation and avoidance of prolonged hospitalization. Many centers around the world have adopted the laparoscopic procedures as the standard procedure to treat HD, but the open approach is inevitable in case of laparoscopic failure[6].

More recently, in view of better cosmetic results and new laparoscopic procedures, De la Torre-Mondragón *et al*[21] described the transanal-endorectal pull-through technique, and Vahdad *et al*[22] described the totally transanal LESS pull-through colectomy. Both procedures are also relatively safe and feasible for short- and long-segment HD. However, besides the ergonomic disadvantages[23,24], the possibility of overstretching the anal sphincter and mesentery of rectosigmoid colon has been reported, which can increase the risk of fecal incontinence[3]. Similar to the single-incision laparoscopy[25-28], these minimally

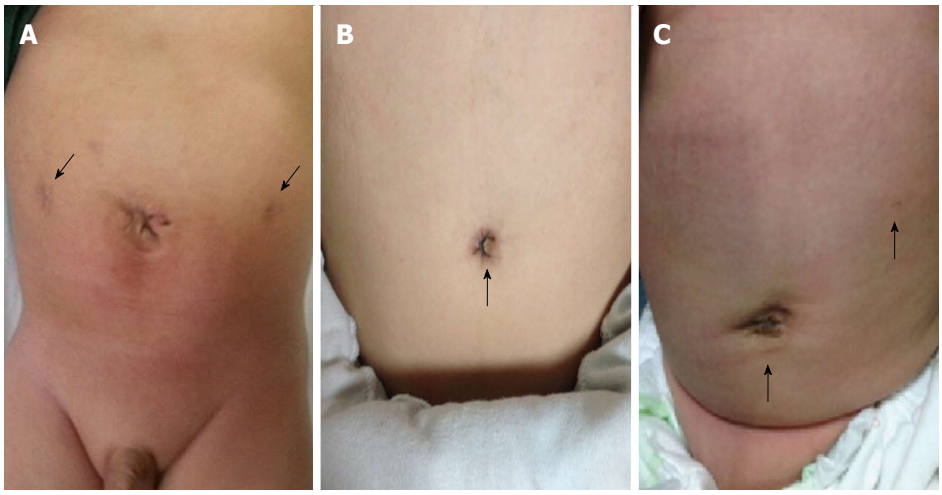

Figure 4 Appearance of abdominal scars on follow-up. A: Conventional laparoscopic pull-through; B: Single-incision laparoscopic pull-through; and C: Hybrid single-incision laparoscopic pull-through procedure.

invasive procedures have excellent cosmetic results and are gaining in popularity.

As for any new technique, there was a learning curve for each of the procedures. In this study, there was no learning curve for the CLP, as a large amount of experience was accumulated by using the CLP[4-6] to treat HD patients. However, the training skill for the SILP procedure was acquired by training on a simulator[29,30] for at least 50 correct attempts before it was successfully applied in real practice. Initially, we had a prolonged operative time among the first five cases, but gradually decreased with the mean operating time to 118 ± 22 min (range: 90-178 min). After overcoming the difficulties encountered in the SILP, the learning curve was rather short for the H-SILP, where training on a simulator for at least ten correct attempts was sufficient because maneuverability was the same as the CLP. We believe that the general operative time did not differ among these procedures because of the proper training, which allowed us to overcome the challenges of the minimally invasive surgery.

With minimally invasive surgery, a man-machine environment is brought into the operating room, which creates mental and physical challenges for the operating team. The science of ergonomics analyzes these challenges and formulates guidelines for creating a work environment that is safe and comfortable for its operators while maintaining effectiveness and efficiency of the process[31]. The ergonomics analysis of the three procedures showed that the operating room, man power, and technical requirements were similar with an experienced operating team and the same coaxial alignments. The only difference in the three approaches was the positioning (Figure 1) of the working ports, which affected the ergonomics of the standard instruments[32]. In this study, curved instruments and a TriPort system were not considered as they are inappropriate for use younger children, especially newborns and infants[33,34].

As the SILP procedure was technically the most challenging, the following findings were observed besides the routine challenges of minimally invasive surgery. Optimum working angles are necessary for suturing and desired tissue manipulation. These working angles are directly influenced by the distance between the working ports. Ideally, good angles for working and suturing are acquired by placing the working ports 10 cm apart outside the body cavity, which provided a working distance of 4 cm inside the body cavity. In the SILP, the distance between the two working ports was approximately 4-5 cm outside the body cavity and the laparoscopic port was situated in the middle. In this context, the working angles were rather restricted and the manipulation was quite difficult.

Relative to the abdomen, each working port projects an internal and an external cone-shaped field, which limits our manipulations to a specific working field perimeter. In the SILP, the interception of these cone-shaped fields narrows the working area and eventually leads to internal and external clashing of the instruments and difficult manipulation. The operative field of vision is affected by the triangulation of the ports with the camera centrally placed. In the SILP, due to one site location of all the working ports, the field of the camera was narrowed, resulting in a restricted field of vision and often clashing of the camera with instruments manipulations (internally and externally). These problems were absent in the CLP and H-SILP, but SILP had the best cosmetic result.

The H-SILP is a "novel" modified version of the SILP. For ergonomic reasons, we shifted the left working port from the umbilical site to the left side the abdomen without trocar. Technically, the maneuverability of the instruments is much easier; similar to the CLP procedure, the working ports were 10 cm apart outside the body cavity, thus improving the triangulation, working angles, and working field. This provided better intra-abdominal exposure with

greater in-line endoscope viewing, greater degrees of freedom, and minimal clashing of the instruments.

We used a trocarless instrument on the left side of the abdomen in the H-SILP to improve the cosmetic result and because there was no indication for interchange of instruments at that site. Although a 5.0 mm or 3.0 mm trocar is less traumatic to the surrounding skin tissue, it leaves a small, remarkable and shiny scar on the abdomen (Figure 4A) compared to a matte and nondistorted scar of a 3 mm trocarless instrument (Figure 4C). In this study, the SILP procedure had the best cosmetic result (scarless), followed by the H-SILP (near scarless appearance) and the CLP (two visible scars), and there was no significant change in the scar score during the entire follow-up period. The scar at the umbilicus was embedded inside, and the appearance was similar to a normal umbilicus, which favors the SILP[35]. The cosmetic result does play an important role in the life of a child, especially in terms of psychologic and psychosocial functioning[36,37].

Ergonomically, the CLP and the H-SILP have the same maneuverability and were less challenging than the SILP. However, with respect to the similar general operative times, we can say that the ergonomic challenges were overcome. Furthermore, to support this statement, the operative times for patients < 1 year of age and short-segment HD patients were not different. So, we can conclude that the SILP is as effective as the CLP and H-SILP. On the other hand, the operative times were significantly different for the patients > 1 year of age and for the patients with long-segment HD. So, we can conclude that the CLP and H-SILP are more convenient for patients > 1 year (toddler, preschool, and school-age patients) and for long-segment HD patients. The most possible reasons for the age and transitional zone ergonomic-related differences are: (1) children > 1 year have a thicker and larger abdominal wall than children < 1 year; (2) the mesenteries and organs are more developed (larger, thicker, and longer); (3) manipulations and dissections are performed at a deeper depth; and (4) for the long aganglionic segment, a longer length of the mesentery are dissected to mobilize the colon to a higher level.

In conclusion, all the procedures are feasible and safe with the same functional outcome. The laparoscopic approach should be selected according to the age, transition zone, and desired cosmetic result. The SILP is more suitable for short-segment HD in neonates and infants. The H-SILP is more convenient for patients > 1 year of age and long-segment HD patients. The CLP can be used in difficult cases where the SILP or the H-SILP might fail.

COMMENTS

Background

Hirschsprung's disease (HD) is one of the diseases responsible for constipation in children. This article describes the main three laparoscopic approaches that the authors have been using to treat HD with the same endorectal, pull-through modified Soave procedure. The authors compared their outcomes and difficulties in order to better understand the choice of one approach over the other regarding patient age, transitional zone, and desired cosmetic result.

Research frontiers

The laparoscopic technique has improved the surgical treatment of HD and has inspired surgeons to undertake more complex laparoscopic procedures to promote early resumption of gastrointestinal function, decrease complications, and to achieve better cosmetic results.

Innovations and breakthroughs

Since 1999, the authors have used a conventional laparoscopic procedure (CLP) to treat HD. In 2010, the authors applied the single-incision laparoscopic procedure (SILP). However, it was more challenging than CLP. In 2011, the authors applied the hybrid single-incision (H-SILP) to preserve the cosmetic advantage of the SILP and the ergonomic advantage of the CLP.

Applications

The authors found that the ergonomic challenges for the SILP procedure were overcome for a specific group of patients. So, with respect to age, transitional zone, and desired cosmetic results, the authors prefer to use: (1) SILP for short-segment HD in neonates and infants; (2) H-SILP for patients > 1 year and long-segment HD patients; and (3) CLP for difficult cases where the SILP or the H-SILP might fail.

Terminology

The transitional zone is the boundary between the ganglionic and aganglionic segment of the colon. Surgically, it is very important to localize the specific affected segment of the colon for the proper amount of bowel resection and to reduce the risk of having to repeat the surgery for remnant aganglionic segments. Laparoscopic/endoscopic surgery is also known as minimally invasive surgery, where the surgeon uses a machine to operate on patients. Ergonomics is an applied scientific analysis of the interaction of man and machine in a specific environment.

Peer-review

This is a well-written paper with a well-performed analysis of three different approaches for the same disease, especially in different patients and those with different localization of the disease.

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ORIGINAL ARTICLE

Observational Study

Helicobacter pylori infection is associated with gallstones: Epidemiological survey in China

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Author contributions: Xu GQ proposed the study; Zhang FM, Chen HT and Hu FL collected the data; Zhang FM, Yu CH, Shen Z and Yuan XP analyzed and interpreted the data; Zhang FM drafted the manuscript; Xu GQ revised the manuscript; all the authors contributed to the design of the study and interpretation of the data, and read and approved the final version to be published.

Institutional review board statement: The study was reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Zhejiang University School of Medicine.

Informed consent statement: All participants were informed verbally about the purpose and design of the study. Written informed consent was not required due to the observational nature of the study. The personal information of each participant was anonymized at collection or prior to analysis.

Conflict-of-interest statement: The authors declared that there is no competing interests in this study.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at xuguoqi@mail.hz.zj.cn. All participants were informed verbally about the purpose and design of the study. Written informed consent was not required due to the observational nature of the study. The personal information of each participant was anonymized at collection and anonymized prior to analysis and the risk of identification is low.

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Abstract

AIM: To elucidate the prevalence and risk factors for gallstones, primarily focusing on *Helicobacter pylori* (*H. pylori*) infection.

METHODS: A total of 10016 Chinese subjects, who had undergone physical examination, fasting ^{13}C urea breath test and abdominal ultrasonography, had sufficient blood test data, and had finished a questionnaire, were included in this cross-sectional study. Participants (n = 1122) who had previous eradication of *H. pylori* were studied separately.

RESULTS: Gallstones were discovered in 9.10% of men and 8.58% of women, with no significant sex difference. Multivariate analyses displayed that age, aspartate aminotransferase, total cholesterol, *H. pylori* infection, hepatitis C virus (HCV) infection, and fatty

liver had a significant association with gallstones (P < 0.05). Successive multiple logistic regression analysis including index of odds ratio (OR) and standardized coefficient (β) indicated that older age (OR/β = 1.056/0.055), *H. pylori* infection (OR/β = 1.454/0.109), HCV infection (OR/β = 1.871/0.123), and fatty liver (OR/β = 1.947/0.189) had a significant positive association with gallstones. After age stratification, *H. pylori* infection and fatty liver still had a significant positive association with gallstones in any age-specific groups, whereas HCV infection had a significant positive association in patients aged > 40 years. The prevalence of gallstones among *H. pylori*-positive, *H. pylori*-eradicated, and *H. pylori*-negative subjects was 9.47%, 9.02%, and 8.46%, respectively. The matched analysis showed that gallstones among *H. pylori* eradicated subjects was significantly lower compared with *H. pylori*-positive subjects (P < 0.05).

CONCLUSION: *H. pylori* infection and fatty liver have a significant positive association with gallstones. *H. pylori* eradication may lead to prevention of gallstones.

Key words: Gallstones; *Helicobacter pylori*; Cross-sectional study

Core tip: Although the pathogenesis of gallstones remains obscure, chronic infection is already accepted as a potential risk factor. There are few large surveys analyzing background factors related to gallstones in Asia. Our study evaluated background factors associated with the presence of gallstones in a cohort of > 10000 subjects, and analysis focusing on the association between *Helicobacter pylori* (*H. pylori*) infection and gallstones in the Chinese population is the most important feature of our study. In this large survey, we found that *H. pylori* eradication may lead to prevention of gallstones, which should shed light on the pathophysiology of gallstones.

Zhang FM, Yu CH, Chen HT, Shen Z, Hu FL, Yuan XP, Xu GQ. *Helicobacter pylori* infection is associated with gallstones: Epidemiological survey in China. *World J Gastroenterol* 2015; 21(29): 8912-8919 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i29/8912.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i29.8912

INTRODUCTION

Gallstones are one of the most prevalent digestive disorders requiring inpatient treatment, as well as a major public health concern worldwide[1]. The prevalence of gallstones in western countries is > 10%[2-4], but in China, they have been rarely reported. The etiology and pathogenesis of gallstones remains obscure. Gallstone formation may be associated with a complex interaction of genetic and environmental factors such as female sex, family history, and ethnicity[5-7]. Lifestyle and some other metabolic disorders also affect gallstone formation, for example, high alcohol consumption, hyperlipidemia, fatty liver, and obesity[1,8-13]. It has also been reported that internal disorders such as hepatitis C virus (HCV) infection, gallbladder polyps and several liver enzymes [aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and γ-glutamyltransferase (GTP)] display a significant association with gallstones[14-17]. In addition, the effect of the gastroduodenal environment is thought to play an important role in the presence of gallstones, and *Helicobacter pylori* are believed to be a mediating factor for gastric and extragastric disease. The gallbladder and bile duct may be two of the targets of chronic *H. pylori* infection. Therefore, we conducted a cross-sectional study to clarify the prevalence and background factors for gallstone formation and investigate the correlation between *H. pylori* infection and gallstones in an attempt to understand the pathogenesis of gallstones and to develop better therapeutic and preventive strategies for this disease.

MATERIALS AND METHODS

Study design and subjects

Subjects were labor union members older than 20 years and retired staff who voluntarily took part in the health examination that included abdominal ultrasonography, fasting ^{13}C urea breath test (^{13}C-UBT), and laboratory data at the International Health Care Center, The First Affiliated Hospital, Zhejiang University School of Medicine, from January 2010 to January 2014. All participants were informed verbally about the purpose and design of the study, and the procedures were approved by the Ethics Committee of Zhejiang University School of Medicine. Those who took proton pump inhibitors, antidiabetic drugs and anti-cholesterol drugs regularly, with a history of cholecystectomy or gastrectomy, were excluded. Participants who had *H. pylori* eradication previously were studied separately.

Physical examination, laboratory assessments and questionnaire

All subjects were instructed to fast overnight and peripheral venous blood samples were collected in the next morning. Laboratory tests, such as serum levels of AST, ALT, ALP, γ-GTP, total bilirubin (T-Bil), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglyceride, total cholesterol (TC), total protein (TP), and hemoglobin concentration, were analyzed. Body mass index (BMI) was calculated as body weight in kilograms divided by the square of the body height in meters. A questionnaire regarding gastrointestinal symptoms,

medical history, lifestyle, and family history was given to all the subjects. Finally, there were questions regarding regular intake of proton pump inhibitors and antidiabetic and anti-cholesterol drugs, and history of *H. pylori* eradication, cholecystectomy or gastrectomy. We categorized smoking and alcohol intake into two groups: nonsmoker *vs* smoker (current or past smoking habit), and higher alcohol intake (often, always) *vs* lower alcohol intake (never, sometimes). HCV infection was defined as positivity of antibodies to HCV (anti-HCV) and without alcohol consumption. The diagnosis of *H. pylori* infection was based on the result of fasting ^{13}C-UBT, and for those who had *H. pylori* eradication history, fasting ^{13}C-UBT was applied at > 1 mo after completion of the standard *H. pylori* eradication therapy. Reference value ranges of all the tested indexes were according to the biochemical criteria of the Department of Clinical Laboratory, The First Affiliated Hospital, Zhejiang University School of Medicine.

Diagnosis of gallstones, fatty liver and gallbladder polyps

Gallstones in the gallbladder and bile duct were diagnosed by abdominal ultrasound. They were diagnosed by the presence of highly reflective echoes from the anterior surface of the stones or movement upon postural change, with or without marked posterior acoustic shadowing. Fatty liver was diagnosed by the following characteristic findings: diffuse increase in hepatic echogenicity with evident contrast between the liver and the kidney; diffuse increase in hepatic echogenicity with blurring of the intrahepatic vessels and the diaphragm; or brightness of the hepatic echogenicity with poor penetration of the posterior hepatic segments, and invisibility of the intrahepatic vessels or diaphragm. The diagnostic criterion for gallbladder polyps was an immobile echo protruding from the gallbladder wall into the lumen, without an acoustic shadow.

Statistical analysis

Statistical analyses were performed with SPSS version 17.0 (SPSS, Chicago, IL, United States). In univariate analyses, continuous data for different groups were presented as mean ± SD, and were compared using Wilcoxon's rank-sum test. Categorical variables were compared with the Pearson's χ^2 test. Logistic regression analyses were used to evaluate the odds ratio (OR) and 95% confidence interval (CI) for gallstones using the related covariates. Standardized coefficient of each variable was calculated using multiple logistic regression analysis, which was applied again after age stratification. Cochran-Armitage test was done in order to determine the effect of *H. pylori* eradication on gallstone prevalence, Finally, a matched pair analysis was performed between the *H. pylori*-positive participants and *H. pylori*-eradicated subjects using McNemar's test with matching criteria of age (± 3), TC (± 1), AST (± 1), condition of fatty liver or anti-HCV.

RESULTS

Subject characteristics

A total of 15523 subjects were enrolled in this study, and we excluded those who did not meet the inclusion criteria. All participants with a history of *H. pylori* eradication were evaluated separately. Thus, 10016 subjects including 4352 men and 5664 women with a mean age of 56.38 ± 14.73 years comprised the primary population (Figure 1). Gallstones were diagnosed in 882 participants (8.81%): 396 men (9.10%) and 486 women (8.58%), with a mean age of 50.50 ± 12.19 years. For the 1122 subjects with a history of *H. pylori* eradication, 124 were still positive for *H. pylori* infection and the other 998 were negative and classified as the *H. pylori*-eradicated group (Figure 1).

Univariate and multivariate analysis evaluating background factors for gallstones

Association of the 13 continuous and seven categorized variables with the prevalence of gallstones was analyzed univariately (Table 1). *H. pylori* infection, anti-HCV, fatty liver, age, BMI, smoking, alcohol intake, γ-GTP, TP, ALP, AST, T-Bil, TC, triglyceride, and LDL-C showed significant association with gallstones ($P < 0.05$), whereas sex, ALT, HDL-C, hemoglobin and gallbladder polyps did not. Expect for sex, all factors were excluded from the performance of multivariate analysis (Table 2), and six demonstrated a significant association with the presence of gallstones. We selected these factors for multiple logistic regression and calculated OR and standardized coefficients (β) (Table 2). Older age (OR/β = 1.056/0.055), *H. pylori* infection (OR/β = 1.454/0.109), anti-HCV (OR/β = 1.871/0.123), and fatty liver (OR/β = 1.947/0.189) had a significant positive association with gallstones. After age stratification, only *H. pylori* infection, fatty liver and anti-HCV still had a significant positive association with gallstones in any age-specific groups.

Effect of H. pylori eradication on gallstone formation

From the multivariate analyses, we found that *H. pylori* infection had a significant positive association with gallstones (Tables 2 and 3); a hypothesis that *H. pylori* eradication might reduce the prevalence of gallstones. Therefore, we compared the above-mentioned 998 subjects who had already undergone successful *H. pylori* eradication therapy to 3410 participants positive for *H. pylori* infection and 6606 negative for *H. pylori* infection (Figure 1). As shown in Table 4, the prevalence of gallstones was 9.47% in the *H. pylori*-positive subjects (without a history of eradication therapy), 9.02% in the *H. pylori*-eradicated subjects, and 8.46% in the *H. pylori*-negative subjects

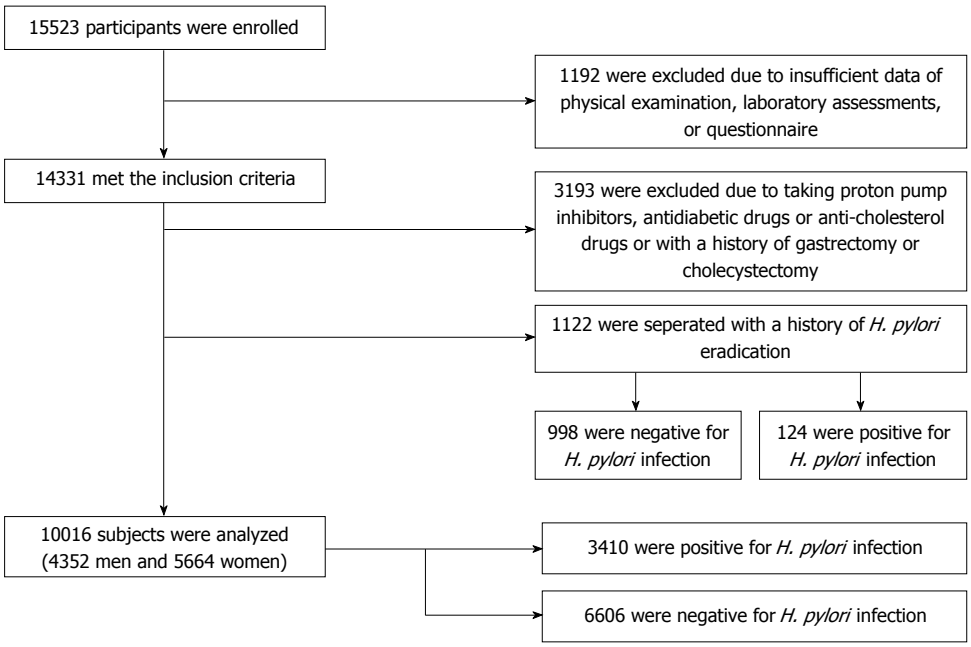

Figure 1 Recruitment flowchart of the study. Healthy adults (n = 15523) attended, and 10016 were analyzed in this study. Subjects who had *H. pylori* eradication previously (n = 1122) were analyzed separately. *H. pylori*: *Helicobacter pylori*.

(without a history of eradication therapy). There was a significant reduction in prevalence of gallstones among subjects with a history of *H. pylori* eradication using the Cochran-Armitage test ($P < 0.0001$). Using the five factors that were significantly associated with the presence of gallstones from multivariate analysis (Table 2), we compared gallstone prevalence between *H. pylori*-positive participants and *H. pylori*-eradicated subjects by matched-pair analyses. Based on age (± 3), TC (± 1), AST (± 1), fatty liver and anti-HCV, the 552 *H. pylori*-positive participants and 552 *H. pylori*-eradicated participants were matched. The prevalence of gallstones was 6.52% and 5.07%, respectively, with a significant difference by McNemar's test ($P = 0.027$).

DISCUSSION

The prevalence of gallstone shows regional variation with higher rates in western countries and lower rates in Asian countries. It was reported that the prevalence of gallstone was 7.9% in men and 16.6% in women in the US, and 29.5% in men and 64.1% in female American Indians[7,18]. In Asian countries, prevalence of gallstones was reported as 6.6% in Singapore, and 5.4% in Thailand[19,20]. Our study found that gallstones accounted for 9.10% of men and 8.58% of women among the 10016 Chinese subjects included (Table 1). Most of the subjects enrolled were white-collar workers or retired staff; they were relatively old, living in good conditions with little movement, and had a high-fat, high-calorie diet and many had asymptomatic gallstones, so they participated in a medical review on a regular basis. All of these factors led to an increase in the incidence of gallstones in our study compared with other regions of China. We believe that the results of health examination may be more representative of the true prevalence of gallstones in the general population of China than hospital-based studies, even autopsy studies, because hospital studies have a relatively limited patient sample and fail to show the true prevalence of gallstones in the general population.

Female gender showed no association with the formation of gallstones, whereas previous studies reported that it had a significant correlation with cholesterol stones, especially in western countries[7,10,21]. Metabolic disorders, such as obesity, diabetes mellitus, or dyslipidemia are common and accepted as risk factors for cholesterol stones[2,3,8,22,23]. Estrogen can increase the cholesterol saturation in bile, which leads to a female predominance of gallstones in western countries. However, in Asian countries such as China, a relative higher proportion of pigment stones are probably observed[24], so the effect of gender distribution may be diminished. In addition, as a result of the national policy of family planning in China since 1982, women were allowed to give birth to only one child and underwent tubal ligation after their first delivery. This led to a reduction in risk factors for gallstone formation, such as being productive and taking oral contraceptives[23,25,26]. Recently, an epidemiological survey from Taiwan showed no gender predominance in gallstone formation but a close association between use of oral contraceptives and gallstones[13]. Therefore, gallstones are mainly attributed to oral contraception rather than gender alone.

Cholesterol stones are the predominant type of gallstones[1,6] and obese people tends to have cholesterol-supersaturated bile and larger gallbladder volume,

Table 1 Characteristics of the 20 variables of the 10016 subjects with or without gallstone

Variables	Presence of gallstone (n = 882)	Absence of gallstone (n = 9134)	P value
Age (yr)	56.38 ± 14.72	46.98 ± 11.56	< 0.001[a]
BMI (kg/m^2)	24.82 ± 3.01	23.77 ± 3.20	< 0.001[a]
ALT (U/L)	25.58 ± 19.67	25.87 ± 31.52	0.386
AST (U/L)	24.14 ± 14.30	24.48 ± 24.43	0.001
ALP (U/L)	67.61 ± 20.02	63.24 ± 17.75	< 0.001[a]
γ-GTP (U/L)	36.52 ± 42.77	25.26 ± 36.02	< 0.001[a]
TC (mmol/L)	4.93 ± 1.07	4.70 ± 1.03	< 0.001[a]
TG (mmol/L)	1.80 ± 3.93	1.52 ± 1.25	< 0.001[a]
TP (g/L)	73.37 ± 4.39	71.77 ± 5.17	< 0.001[a]
T-Bil (umol/L)	13.63 ± 6.67	12.44 ± 5.79	< 0.001[a]
HDL-Chol (mmol/L)	1.18 ± 0.37	1.31 ± 2.41	0.091
LDL-Chol (mmol/L)	2.85 ± 6.65	2.60 ± 2.04	0.012
Hb (g/L)	144.81 ± 18.11	148.77 ± 18.30	0.721
H. pylori, *n* (%)			< 0.001[a]
Positive	323 (9.47)	3087 (90.53)	
Negative	559 (8.46)	6047 (91.54)	
Alcohol intake			< 0.001[a]
Yes	545 (10.88)	4463 (89.12)	
No	337 (55.89)	4671 (44.11)	
Smoking			< 0.001[a]
Yes	475 (13.59)	3021 (86.41)	
No	407 (6.24)	6113 (93.76)	
Gender			0.095
Men	396 (9.10)	3956 (90.90)	
Women	486 (8.58)	5178 (91.42)	
Anti-HCV			
Positive	526 (11.98)	3865 (88.02)	0.001[a]
Negtive	356 (6.33)	5269 (93.67)	
Fatty liver			
Presence	572 (13.05)	3811 (86.95)	< 0.001[a]
Absence	310 (5.50)	5323 (94.50)	
GB polyp			
Presence	402 (10.71)	3350 (89.29)	0.197
Absence	480 (7.67)	5784 (92.33)	
Total	882 (8.51)	9134 (92.49)	

Data were expressed as mean ± SD of each variable. By applying the Wilcoxon analysis, P values of the 13 continuous variables were calculated. By applying Pearson's χ^2 test, P values of the seven categorized variables were calculated. [a]P < 0.05, Presence *vs* Absence. BMI: Body mass index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; γ-GTP: Gamma glutamyltransferase; TC: Cholesterin; TG: Triglyceride; TP: Total protein serum; T-Bil: Total bilirubin; HDL-Chol: High density lipoprotein cholesterol; LDL-Chol: Low density lipoprotein cholesterol; Hb: Hemoglobin concentration; *H. Pylori*: *Helicobacter pylori*; Anti-HCV: Antibodies to HCV; GB polyp: Gallbladder polyp.

thus, dyslipidemia in obese people is a likely cause of gallstones. We discovered that high TC level appears to be related to gallstones in our multivariate analysis, but the association was not significant in multiple logistic regression analysis (Table 2). Moreover, many studies could not confirm the relationship between dyslipidemia and the presence of gallstones[13,27]. Dyslipidemia may contribute to the formation of gallstones, especially cholesterol stones, but the relationship between dyslipidemia and gallstones remains inconclusive[28]. It is reported that the mechanism and risk factors for gallstones differ among stone types. Thus, future studies should focus on the different types of gallstones.

Many reports have confirmed that BMI is an important risk factor for gallstones[7,29], but an inverse relationship between BMI and gallstones was shown in our analysis. BMI may help to establish whether a person is fat or thin, but it does not make clear a person's fat percentage. The formation of gallstones may be more related to abdominal circumference, so other indicators, such as waist-to-hip ratio may be more meaningful[30].

It has been shown that HCV RNA can colonize gallbladder cells, impair gallbladder epithelium lipid absorption, and alter gallbladder mucosal function[31-33]. Moreover, HCV binds to apolipoprotein A1 and leads to liver steatosis and chronic hepatitis[34]. All of these factors contribute to gallstone formation. Fatty liver is related to increasing prevalence of gallstones due to insulin resistance and visceral obesity[12,13,35]. We found that HCV infection and fatty liver had a strong association with gallstones.

Age plays an important role in the prevalence of *H. pylori* infection, and was also a significant factor influencing the presence of gallstones in our study. In order to avoid selection bias of age, we evaluated the association between gallstones and *H. pylori* infection by age stratification. After age stratification, *H. pylori* infection and fatty liver still had a significant positive association with gallstones in age-specific groups, whereas HCV infection had a strong association in those aged > 40 years.

H. pylori can cause many gastroduodenal diseases such as atrophic gastritis, peptic ulcer, gastric carcinoma and mucosa-associated lymphoid tissue lymphoma[36,37]. Recently, it has been reported that *H. pylori* is also associated with extragastric diseases, such as thyroid nodules, metabolic syndrome, nonalcoholic fatty liver disease, insulin resistance and autoimmune diseases[38-41]. DNA, RNA and antigens specific for *H. pylori* were repeatedly detected in bile, biliary tract tissue and stone specimens[42,43]. Hence, a hypothesis that *Helicobacter* species are etiological agents in gallstone formation has been suggested. Our study showed a positive association between *H. pylori* infection and gallstones in humans by analyzing a large cohort of 10 016 adults (Tables 1 and 2). After age stratification, *H. pylori* infection was still a risk factor for the prevalence of gallstones (Table 3). Analysis of those who had successful *H. pylori* eradication also supported that a state of chronic infection promotes gallstone formation (Table 4), as was reported in a recent large-scale survey with a cohort of > 10000 subjects in Japan[44]. Accordingly, we think that *H. pylori* infection is a risk factor for gallstone formation in humans, although we lack details of the precise mechanism.

There were some limitations to our study. The first limitation was that the sample was not sufficiently representative. Study subjects were those who participated in health screening in the International Health Care Center, The First Affiliated Hospital of

Table 2 Multivariate analysis and mutiple logistic analysis of the correlated variables for gallstone

Variables	Multivariate analysis			Mutiple logistic analysis		
	β	*P* value	OR (95%CI)	β	*P* value	OR (95%CI)
Age	0.056	0.009[a]	1.057 (1.045-1.070)	0.055	< 0.001[a]	1.056 (1.043-1.070)
Gender (female)	-1.048	< 0.001[a]	0.351 (0.265-0.770)			
BMI	-0.050	0.105	0.951 (0.845-1.077)			
ALP	-0.001	0.610	0.999 (0.839-1.152)			
AST	0.712	< 0.001[a]	1.048 (1.045-1.092)			
γ-GT	0.002	0.260	1.002 (1.045-1.070)			
TC	0.017	0.006[a]	1.018 (1.006-1.029)			
TG	-0.113	0.007[a]	0.893 (0.595-0.982)			
T-Bil	-0.044	< 0.001[a]	0.957 (0.856-1.081)			
LDL-Chol	0.001	0.946	1.001 (0.901-1.023)			
TP	-0.108	< 0.001[a]	0.898 (0.601-0.994)			
Helicobacter pylori positive	0.588	< 0.001[a]	1.800 (1.386-2.337)	0.109	< 0.001[a]	1.454 (1.102-2.521)
Anti-HCV	1.320	< 0.001[a]	3.742 (1.426-5.217)	0.123	< 0.001[a]	1.871 (1.441-3.681)
Smoking	-0.741	< 0.001[a]	0.477 (0.295-0.692)			
Alcohol intake	-0.124	0.037	0.883 (0.421-0.911)			
Fatty liver	1.339	< 0.001[a]	3.814 (1.886-6.023)	0.189	< 0.001[a]	1.947 (1.212-3.987)

By applying logistic regression analysis, *P* values were calculated. [a]$P < 0.05$. β: Partial regression coefficient; SE: Standard error of partial regression coefficient.

Table 3 Positively correlated variables for gallstone formation stratified by age

Variables	Age (20-39 yr)		Age (40-64 yr)		Age (≥ 65 yr)	
	OR	*P* value	OR	*P* value	OR	*P* value
Gender (female)	0.658	0.054	0.905	0.062	0.714	0.038[a]
Helicobacter pylori positive	1.521	0.006[a]	1.510	0.015[a]	1.324	< 0.001[a]
Anti-HCV	0.988	0.004[a]	1.598	0.019[a]	1.563	0.002[a]
Fatty liver	1.582	0.003[a]	1.922	< 0.001[a]	1.752	< 0.001[a]
TC	1.025	0.921	1.520	0.102	0.914	0.023[a]
AST	0.382	0.221	1.249	0.024	1.547	< 0.001[a]

P values calculated using muitiple logistic regression analysis. [a]$P < 0.05$.

Table 4 Prevalence of gallstone in *Helicobacter pylori*-positive, -eradicated, and -negative subjects *n* (%)

	Presence of gallstones	Absence of gallstones
Helicobacter pylori positive (without eradication)	323 (9.47)	3087 (90.53)
Helicobacter pylori negative after eradication	90 (9.02)	908 (90.98)
Helicobacter pylori negative (without eradication)	559 (8.46)	6047 (91.54)

Cochran–Armitage test, $P < 0.0001$, Presence *vs* Absence.

Zhejiang University School of Medicine. Most of them were white collar workers or retired staff who were older in age, in good living conditions, with a high-fat, high-calorie diet, and lack of exercise. People of lower economic status were not represented in our study, which could have led to selection bias and an increase in the incidence of gallstones. The second limitation was that a cause-and-effect relationship could not be elucidated due to the inherent limitation of a cross-sectional study. Both *H. pylori* infection and gallstones are common disorders worldwide, therefore, it is of importance to clarify whether *H. pylori* eradication can prevent gallstones. The final limitation was the lack of control for potentially confounding risk factors, such as fasting plasma glucose, oral contraceptives, and cirrhosis. We are planning a randomized prospective study to determine the effect of *H. pylori* eradication on gallstone formation. Most importantly, long-term follow-up of the *H. pylori*-positive group, *H. pylori*-eradicated group and *H. pylori*-negative group (Table 4) should verify the present conclusion that *H. pylori* infection increases the risk of gallstones and *H. pylori* eradication may lead to prevention of gallstones. Further studies are also necessary to understand thoroughly the mechanisms mediating this relationship and help to clarify the key point of disease prevention.

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ultrasonographic examination in this study.

COMMENTS

Background

Gallstones are one of the most prevalent digestive disorders and often require surgical management. Although the pathogenesis of gallstones remains obscure, chronic infection is already accepted as a potential risk factor. *Helicobacter pylori* (*H. pylori*) is detected in bile, biliary tract tissue and stone specimens, and it has been clearly demonstrated that *H. pylori* promotes gallstone formation in animal and human research. In recent decades, a few large surveys have been performed, analyzing background factors related to gallstones from Europe, North America and Japan. Thus, it is necessary to conduct a large epidemiological study in China to evaluate background factors associated with the presence of gallstones, especially focusing on *H. pylori* infection.

Research frontiers

To date, four large-scale studies from Europe, North America and Japan analyzing background factors related to gallstones are well known: MICOL study investigating 29584 individuals (15910 men and 13674 women) from Italy found that increasing age and body mass index and a maternal family history of gallstone disease were the most consistent associations. The third NHANES survey analyzing 14238 Americans (6688 men and 7550 women) revealed that > 20 million persons had gallbladder disease in the US. Ethnic differences in gallbladder disease prevalence differed according to sex and were only partly explained by known risk factors. Swedish Twin Registry studies investigating 43141 or 58402 twin pairs in Sweden showed positive associations between BMI and the development of symptomatic gallbladder disease, high alcohol consumption was associated with a decreased risk against gallbladder disease, tobacco use had no impact on gallbladder disease. Yu Takahashi designed the research of 15551 subjects comprised of 8625 men and 6926 women, displaying that *H. pylori* infection is positively associated with gallstones. *H. pylori* eradication may lead to prevention of gallstones.

Innovations and breakthroughs

A recent study detected various *Helicobacter* species in bile, biliary tract tissue and stone specimens, which indicated the role of *H. pylori* in the formation of gallstones. There are few large surveys with a cohort of > 10000 subjects worldwide, and such a large study focusing on the relationship between *H. pylori* infection and gallstone formation has not been performed in China. By analysis of the large cohort of > 10000 adults, this study showed for the first time a positive association between *H. pylori* infection and presence of gallstones, and found that *H. pylori* eradication may lead to prevention of gallstones in Chinese people.

Applications

H. pylori infection had a significant positive association with gallstones in any age-specific groups. *H. pylori* eradication may reduce the prevalence of gallstones. Thus, positive treatment of *H. pylori* infection may represent a new method of treating or preventing gallstones.

Terminology

H. pylori is a Gram-negative microaerophilic microorganism that can cause many gastroduodenal diseases such as atrophic gastritis, peptic ulcer, gastric carcinoma and mucosa-associated lymphoid tissue lymphoma, as well as extragastric diseases such as thyroid nodules, nonalcoholic fatty liver disease, and autoimmune diseases.

Peer-review

The title is adequate to reveal the purpose of the study. The study was well designed and conducted. The conclusions are in accord with the results. Statistical analysis and conclusions are adequate. Limitations and future prospect are presented.

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ORIGINAL ARTICLE

Prospective Study

Viral hepatitis prevalence in patients with active and latent tuberculosis

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Abstract

AIM: To assess the prevalence of hepatitis B virus (HBV) and hepatitis C virus (HCV) infection and association with drug induced liver injury (DILI) in patients undergoing anti-tuberculosis (TB) therapy.

METHODS: Four hundred and twenty nine patients with newly diagnosed TB - either active disease or latent infection - who were due to commence anti-TB therapy between September 2008 and May 2011 were included. These patients were prospectively tested for serological markers of HBV, HCV and human immunodeficiency virus (HIV) infections - hepatitis B core antigen (HBcAg), hepatitis B surface antigen (HBsAg), hepatitis B e antigen, IgG and IgM antibody to HBcAg (anti-HBc), HCV IgG antibody and HIV antibody using a combination of enzyme-linked immunosorbent assay, Western blot assay and polymerase chain reaction techniques. Patients were reviewed at least monthly during the TB treatment initiation phase. Liver function tests were measured prior to commencement of anti-TB therapy and 2-4 wk later. Liver function tests were also performed at any time the patient had significant nausea, vomiting, rash, or felt non-specifically unwell. Fisher's exact test was used to measure significance in comparisons of proportions between groups. A *P* value of less than 0.05 was considered statistically significant.

RESULTS: Of the 429 patients, 270 (62.9%) had active TB disease and 159 (37.1%) had latent TB infection. 61 (14.2%) patients had isolated anti-HBc positivity, 11 (2.6%) were also HBsAg positive and 7

(1.6%) were HCV-antibody positive. 16/270 patients with active TB disease compared to 2/159 patients with latent TB infection had markers of chronic viral hepatitis (HBsAg or HCV antibody positive; $P = 0.023$). Similarly the proportion of HBsAg positive patients were significantly greater in the active *vs* latent TB infection group (10/43 *vs* 1/29, $P = 0.04$). The prevalence of chronic HBV or HCV was significantly higher than the estimated United Kingdom prevalence of 0.3% for each. We found no association between DILI and presence of serological markers of HBV or HCV. Three (5.3%) patients with serological markers of HBV or HCV infection had DILI compared to 25 (9.5%) patients without; $P = 0.04$.

CONCLUSION: Viral hepatitis screening should be considered in TB patients. DILI risk was not increased in patients with HBV/HCV.

Key words: Epidemiology; Hepatitis B; Hepatitis C; Tuberculosis; Drug induced liver injury

Core tip: Tuberculosis (TB) patients are not routinely tested for viral hepatitis in the United Kingdom. This is the first study from a European centre investigating the prevalence of hepatitis B virus (HBV) and hepatitis C virus (HCV) in patients with TB. We found that chronic HBV and HCV prevalence in TB patients were 9 and 5 times greater than the estimated United Kingdom prevalence respectively. We also found that a significantly greater proportion of patients with active TB had chronic Hepatitis B compared with patients with latent TB infection. In our study there was no association between drug induced liver injury risk and presence of serological markers of HBV/HCV.

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INTRODUCTION

Tuberculosis (TB) is the leading cause of death from a curable infectious disease; in 2010, 1.45 million people died from TB and there were 8.8 million incident cases[1]. In the United Kingdom, the incidence of TB has steadily increased over the past two decades[2]. 7892 cases were reported in 2013, the majority in urban areas, with London accounting for 38% of those cases[2]. Over 80% of cases are non-United Kingdom born, and rates of TB in the non-United Kingdom born are approximately twenty-fold higher (86/100000) than those born in United Kingdom (4/100000)[2].

Standard four-drug anti-TB therapy for active disease (isoniazid, rifampicin, pyrazinamide and ethambutol) is associated with a range of significant side effects, the most serious of which is drug induced liver injury (DILI), which carries a mortality rate of up to 5%[3,4]. All anti-TB medications are potential causes of DILI[5]. The incidence of DILI from anti-TB treatment has been variably reported - between 2% and 28% - with a number of factors, including HLA phenotype and ethnicity, having been found to alter an individual's risk for a hepatotoxic drug reaction[5,6]. The clinical presentation of DILI ranges from transient mild elevation of liver enzymes to fulminant liver failure, and a commonly used definition of DILI is an increase in serum alanine transaminase (ALT) greater than 3 or 5 times the upper limit of normal (ULN) with or without symptoms of acute hepatitis respectively[4].

The World Health Organization (WHO) estimates approximately 240 million people worldwide are chronically infected with hepatitis B virus (HBV)[7]. Areas of high prevalence are similar to the global TB epidemiological "hotspots" and include sub-Saharan Africa and South Asia, where the prevalence is estimated to be between 8 and 20%[8]. The WHO estimate 3% of the World's population are infected with hepatitis C virus (HCV), with 170 million being chronic carriers[9]. European countries report a prevalence of HCV in the general population of between 0.5%-2% and global areas of high prevalence again include Africa, particularly Egypt, and Asia[9]. In 2011 it was estimated that around 216000 individuals were the United Kingdom are chronically infected with HCV[10], and the HPA and WHO estimate the United Kingdom prevalence of chronic HBV infection to be similar to this, at 0.3%. Chronic infection with HBV and/or HCV can cause progressive liver fibrosis and cirrhosis, liver failure and liver cancer. Effective therapies exist for both these viruses if they are diagnosed before advanced liver disease occurs, and early detection and treatment is important in minimising the health burden associated with chronic HBV and HCV. However, both these viruses are relatively asymptomatic and hence international liver associations recommend screening for HBV and HCV in high risk groups[11,12].

Several studies report an increased prevalence of viral hepatitis infection in TB patients[14-17] (Table 1). However, none of these studies have been performed in a European setting, so their applicability to the United Kingdom is not known. Although results are not universal, a number of international studies suggest that co-existing viral hepatitis may be a significant risk factor for DILI[13,18-22]. No studies on the prevalence of viral hepatitis in patients undergoing anti-TB therapy and the risk they add to DILI have been carried out in the United Kingdom or Europe, which have seen an increase in TB cases over recent years.

Table 1 Prevalence of hepatitis B virus and hepatitis C virus infection in tuberculosis patients in other studies *n* (%)

Study (country)	Study population	Number of patients	HBsAg	HBsAg or anti-HBc	Anti-HCV
Kuniholm *et al*[14] (Georgia)	All active TB patients	300	13 (4.3)	27 (9)	36 (12)
Blal *et al*[15] (Brazil)	All active TB patients	209	6 (2.8)	56 (26.8)	-
Sirinak *et al*[16] (Thailand)	HIV infected active TB patients	849	70 (9)	-	237 (31)
Aires *et al*[17] (Brazil)	All active TB patients	402	13 (3.2)	103 (25.6)	-

HBsAg: Hepatitis B surface antigen; HCV: Hepatitis C virus; HBc: Hepatitis B core; TB: Tuberculosis.

United Kingdom patients infected with TB are offered human immunodeficiency virus (HIV) screening[23] due to an increased prevalence of co-infection, but viral hepatitis screening is not routinely offered and might be of value if the background prevalence of viral hepatitis is significant in patients with TB infection. London is a multicultural city with a significant number of immigrants from high endemic TB countries, suggesting that the prevalence of viral hepatitis infection may be higher than suspected.

MATERIALS AND METHODS

Recruitment

We recruited patients with newly diagnosed TB - either active disease or latent infection - at St. Mary's Hospital, Imperial College Healthcare NHS Trust, London, who were due to commence standard anti-TB therapy for either stages of the infection between September 2008 and May 2011. The inclusion criteria were: newly diagnosed TB (active or latent); 18 years of age or above; ability to give informed consent; and no known history of chronic liver disease, viral hepatitis or HIV. No patients were immunosuppressed. Research ethics approval was obtained (No. 10/H0709/44).

Clinical phenotyping

TB infection was confirmed using the standard protocols in our clinic. Active TB infection was defined by clinical and radiological characteristics of symptomatic TB infection, with or without confirmatory culture information. If culture negative, then the patient was deemed to have had active TB if they made a satisfactory response to anti-tuberculosis therapy.

Latent TB infection was defined as an asymptomatic patient without radiographic evidence of active TB disease, and with a positive tuberculin skin test read at 48-72 h by an experienced nurse (defined as > 5 mm induration if not BCG vaccinated or ⩾ 15 mm if BCG vaccinated), subsequently confirmed with a positive interferon gamma release assay [either T.Spot. *TB* (Oxford Immunotec, Oxford, United Kingdom), or Quantiferon Gold-in-Tube (Cellestis Ltd, Victoria, Australia)].

Drug treatment

For active disease, standard anti-tuberculous therapy in our clinic was rifampicin 600 mg/d (R), Isoniazid 300 mg/d (H), Pyrazinamide 25 mg/kg per day (Z) (these three jointly given as Rifater with the number of tablets dependent on weight; if the weight was sufficiently low, then the drugs were given separately as per guidelines[23]), and Ethambutol 15 mg/kg per day (E) for 2 mo; followed by continuation rifampicin and isoniazid for a further 4 mo [8 mo if central nervous system (CNS) involvement]. Treating clinicians had the option to extend the initiation phase to 3 mo if the patient was persistently smear positive after one month of therapy and could extend the total duration of therapy if they felt the initial burden disease was high, on a case by case basis. Adjuvant steroid therapy was used in patients with pericardial or CNS disease, as per national guidelines[23] and weekly pyridoxine 50mg orally was administered to all cases. Drug resistant cases were treated according to culture results as per national guidelines[23].

For latent infection, patients were treated with 3 mo of Rifampicin 600mg daily (R) and Isoniazid 300 mg daily (H) (jointly given as Rifinah-300, two tablets daily), in keeping with standard United Kingdom practice.

Data collection

Patients' demographic, serological and clinical data were collected following informed consent, from the London TB register, hospital clinical databases and clinical notes. Demographic information collected included age, gender and ethnicity. In addition to biochemical liver function tests, the following serological results were also collected: HIV antibody, hepatitis B core antigen (HBcAg), hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg), IgG and IgM antibody to HBcAg (anti-HBc) and HCV IgG antibody (anti-HCV).

As per standard clinical protocol, patients were reviewed at least monthly during the TB treatment initiation phase. Liver function tests, including alanine transaminase (ALT) levels, were measured prior to commencement of anti-TB therapy and again 2-4 wk later. An ALT level of 40 IU/mL was taken as the upper limit of normal (ULN). DILI was defined as an ALT of greater than 80IU/ml (2 × ULN), as defined by the Council for International Organisation of Medical Sciences (CIOM) DILI diagnostic scale[24] with no other apparent cause for abnormal liver biochemistry. Severe DILI was defined at an ALT of 5 × ULN. Liver

Table 2 Demographic and Serological data of patients *n* (%)

Characteristic	Patients				
	Total	HBV/HCV Negative	HBsAg	Isolated Anti-HBc (IgG)	HCV (IgG)
Female	209 (48.7)	173 (40.3)	4 (0.9)	31 (7.2)	1 (0.2)
Male	220 (51.3)	177 (41.3)	7 (1.6)	30 (7.0)	6 (1.4)
Age (yr)					
18-35	155 (36.1)	132 (30.8)	0 (0.0)	21 (4.9)	2 (0.5)
36-53	133 (31.0)	106 (24.7)	7 (1.6)	16 (3.7)	4 (0.9)
54-71	88 (20.5)	67 (15.6)	3 (0.7)	17 (4.0)	1 (0.2)
72-90	51 (11.9)	43 (10.0)	1 (0.2)	7 (1.6)	0 (0.0)
> 90	2 (0.5)	2 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)
Ethinicity					
Indian Subcontinent	152 (35.4)	132 (30.8)	1 (0.2)	18 (4.2)	1 (0.2)
Black African (Sub Saharan)	104 (24.2)	73 (17.0)	6 (1.4)	23 (5.4)	2 (0.5)
Black Caribbean	21 (4.9)	17 (4.0)	0 (0.0)	4 (0.9)	0 (0.0)
North African	25 (5.8)	20 (4.7)	0 (0.0)	5 (1.2)	0 (0.0)
South East Asia	34 (7.9)	26 (6.1)	3 (0.7)	5 (1.2)	0 (0.0)
White all other (European/American/Australian)	46 (10.7)	38 (8.9)	1 (0.02)	5 (1.2)	2 (0.5)
White South American	15 (3.5)	15 (3.5)	0 (0.0)	0 (0.0)	0 (0.0)
White UK	32 (7.5)	29 (6.8)	0 (0.0)	1 (0.02)	2 (0.5)
Total	429	350 (81.6)	11 (2.6)	61 (14.2)	7 (1.6)

HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HBc: Hepatitis B core.

function tests (LFTs) were performed at any time the patient had significant nausea, vomiting, rash, or felt non-specifically unwell.

HIV, HBV and HCV serological tests were performed at St Mary's hospital pathology department. Enzyme-linked immunosorbent assay (ELISA) was used for detection of anti-HIV antibodies and confirmed by Western blot assay. Screening for HBV and HCV was conducted using second generation ELISA and confirmatory tests for HBsAg or HCV positive results included polymerase chain reaction techniques. Data were analysed using Prism 5 (GraphPad Software Inc.). Fisher's exact test was used to measure significance in comparisons of proportions between groups. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Demographics

A total of 487 patients were recruited. 58 patients were excluded due to absent results or loss to follow up. Of the remaining 429 patients, 270 (62.9%) patients had active TB disease and 159 (37.1%) had latent TB infection. The mean age was 46.4 years and 51.3% of patients were male. The majority of patients were from the Indian Subcontinent and Sub Saharan Africa, together accounting for 59.7% of patients. 7.5% of patients were White British (Table 2).

Prevalence of HCV/HBV markers

Of 429 patients screened, 79 (18.4%) had positive serological markers for HBV or HCV infection. 61 (14.2%) had isolated anti-HBc antibody only; 11 (2.6%) were also HBsAg positive and 7 (1.6%) were positive for HCV IgG antibody. No patients had HBV/HCV co-infection (Table 2). All patients positive for HBsAg and HCV Antibody were previously unknown to have a history of viral hepatitis.

Active TB disease vs latent TB infection

A similar proportion of patients in both groups had serological markers for HBV or HCV infection (49/270 active TB disease patients *vs* 30/159 latent TB infection patients; $P = 0.9$, Fisher's exact test) (Table 3).

However, there was a significant difference between these groups with respect to chronic viral hepatitis: 16/270 patients with active TB disease compared to 2/159 patients with latent TB infection had markers of chronic viral hepatitis (HBsAg or HCV antibody positive; $P = 0.023$, Fisher's exact test). Similarly, while there was no difference between the groups with respect to anti-HBc antibody positivity (43/270 in active TB disease group *vs* 29/159 in latent TB infection group; $P = 0.59$, Fisher's exact test), the proportion of this group whose HBsAg was positive was significantly greater in the active TB patient group (10/43 in active TB patients *vs* 1/29 in latent TB infection group; $P = 0.04$, Fisher's exact test).

A greater proportion of active TB disease patients were HCV seropositive compared to latent TB infection patients although this difference was not significant (6/270 active TB disease patients *vs* 1/159 latent TB infection patients; $P = 0.27$, Fisher's exact test).

Viral hepatitis and HIV co-infection

Fifteen patients were already known to be HIV positive and a further 260 patients consented to HIV screening (245 active TB disease, 30 latent TB infection). Eleven patients were newly diagnosed with HIV meaning that, of the 275 patients, 26 (9.5%) were infected with HIV. Six (23.1%) of the HIV positive patients were co-

Table 3 Prevalence of hepatitis B virus and hepatitis C virus infection in active TB disease vs latent tuberculosis infection patients *n* (%)

HBV/HCV infection	Patients		*P* value (Fisher's exact test)
	Active	Latent	
HBsAg/HCV Ab both negative	221 (81.9)	129 (81.1)	0.9
HBsAg or HCV Ab positive	49 (18.1)	30 (18.9)	
HCV (IgG) positive	6 (2.2)	1 (0.6)	0.27
Anti-HBc (IgG) positive	43 (12.2)	29 (17.6)	0.59
Of these: HBsAg positive	> 10 (23.2)	> 1 (3.4)	> 0.04

HBsAg: Hepatitis B surface antigen; HCV: Hepatitis C virus; HBc: Hepatitis B core.

infected with HBV or HCV (P = 0.26, Fisher's exact test).

Pre-treatment ALT Levels

Pre-treatment ALT levels were available in 399 patients, 71 of whom were HCV or anti-HBc positive (including those also positive for HBsAg). Fourteen (19.7%) of these patients had ALT levels of greater than 40 IU/mL compared to 61 (18.6%) patients negative for markers of HBV or HCV (P = 0.87, Fisher's exact test). 13 (3.3%) patients had ALTs of greater than 2 × ULN (> 80 IU/mL), 3 of whom had ALTs of greater than 5 × ULN (> 200 IU/mL). All of these patients were negative for all markers for HBV and HCV.

Post-treatment ALT Levels

Post-treatment ALTs were documented in 396 patients, 72 of whom were HCV or anti-HBc positive (including those also positive for HBsAg). 16 (22.2%) of these patients had ALT elevation > 40 IU/mL compared to 89 (27.5%) patients negative for markers of HBV or HCV (P = 0.31, Fisher's exact test). Six (8.3%) anti-HBc or HCV positive patients had ALT > 80 IU/mL compared to 33 (10.2%) patients without serological evidence of HBV or HCV (P = 0.83, Fisher's exact test). Of the 13 patients with ALT > 200 IU/mL, only one was positive for HBV or HCV serological markers (P = 0.48, Fisher's exact test).

ALT levels post-treatment following normal pre-treatment levels

Three hundred and twenty patients had normal (< 40 IU/mL) pre-treatment ALTs and also had follow-up LFTs; of these, 57 were positive for serological markers HBV or HCV. ALT elevation of greater than 80 IU/mL was seen in 3 (5.3%) patients with positive HBV or HCV serological markers, compared to 25 (9.5%) patients negative for serological markers HBV/HCV (P = 0.44, Fisher's exact test). Twelve patients had ALT > 200 IU/mL of whom only one had serological evidence of HBV/HCV (P = 0.70, Fisher's exact test). Five patients required interruption of treatment due to significantly elevated LFTs, none of whom had serological evidence of HBV/HCV. Overall, 13 (46.4%) of the 28 patients with DILI were above the age of 50 and 14 (50%) were male.

DISCUSSION

This is the first study from a European centre to investigate the prevalence of HBV and HCV in patients with TB. We found that 18.4% of newly diagnosed TB patients at a central London teaching hospital had markers of HBV or HCV; 2.6% of patients were HBsAg positive and 1.6% were anti-HCV Ab positive. All these diagnoses were new, and led to specialist referral with the aim of preventing long-term complications of chronic viral hepatitis.

Our study found that chronic HBV prevalence in TB patients was almost 9 times greater than the estimated overall United Kingdom prevalence. Similarly, the prevalence of HCV amongst TB patients in our study was over 5 times greater than the estimated United Kingdom prevalence of HCV. The majority of infected patients were of Indian Subcontinent or Black African origin, and our distribution of ethnicities was representative of London's multi-racial TB population[25].

A recent study by Uddin and colleagues looked at the prevalence of HBV and HCV in South Asian immigrants in England attending community centres[26]. 4998 individuals were screened and HBsAg or anti-HCV Ab was present in 1.2% and 1.6% of patients respectively, again higher than the national estimated prevalence. This may be partially explained by the older demographic screened in this study - attending mosques and temples - who are more likely to have been born and raised in South Asia and hence would be at a greater risk of acquiring infection with HBV/HCV at younger ages. The prevalence of chronic hepatitis B in our study was more than twice that found in the study by Uddin *et al*[26], reflecting the wider ethnic background to our population, and suggesting that screening for HBV and HCV may be more effective in health care settings which might capture a wider range of infected individuals.

Although HIV and HBV/HCV have similar risk factors, we found no significant association between these infections in our cohort of TB patients, suggesting that HIV seropositivity alone would not identify patients with unknown HBV/HCV.

Of note, a significantly greater proportion of patients with active TB had chronic Hepatitis B compared with patients with latent TB infection. The proportion of patients in the latent and active groups positive for anti-HBc did not differ, suggesting that whilst both groups had equal exposure to the virus, the group with active TB were more likely to fail to clear virus and remain sAg positive. This could be attributable to the relatively small number of latent TB patients in the study; alternatively, there may be shared pa-

thways of immune control, perhaps involving MHC I-restricted CD8 T cells important in both viral[27] and mycobacterial[28] control, which could link immune dysregulation in both Hepatitis B viral infection and M. tuberculosis infection.

It is relevant to note that, on further follow-up, none of the patients with HBsAg and/or Anti HCV positivity had cirrhosis, as chronic liver disease itself is a potential risk factor for acquiring TB.

Different studies have used varying definitions of hepatotoxicity; our study employed a range of thresholds, including one which is lower than the ATS guidelines on hepatotoxicity[29], with the aim of providing a more comprehensive analysis of the derangement in liver function that might occur in this population. Our study followed a standard protocol whereby LFTs were measured pre-treatment and 2-4 wk after treatment. Further LFTs were only measured if the patient had abnormal levels at week 2-4 or developed symptoms; it is therefore possible that patients developing asymptomatic DILI after 2-4 wk of treatment may have been missed, although this is not common[29]. As with a number of other studies, we found no association between HBV/HCV seropositivity and DILI at both a lower and a higher threshold for diagnosis[5,16,30]; suggesting that anti-TB medication can generally be safely administered to those infected with HBV/HCV in settings similar to ours, provided liver function is regularly monitored. Although DILI tends to occur early in the course of anti-TB therapy, the prevalence of DILI in our study may potentially be underestimated as follow-up liver function tests were only checked once, unless clinically indicated.

It is also important to note that over 14% of patients undergoing anti-TB treatment were positive for anti-HBc. Without prophylactic anti-viral therapy, these patients are at risk of HBV reactivation and liver failure if they are immunosuppressed or undergo immunomodulatory therapy or chemotherapy in the future.

Without early detection and appropriate management, chronic infection with HBV or HCV can lead to cirrhosis, liver failure and liver cancer. The Health Protection Agency (United Kingdom) advises HCV screening in intravenous drug users (IVDU) and men who have sex with men[10] and the ATS recommends viral hepatitis screening in TB patients with risk factors such as IVDU, those born in endemic areas and in HIV positive individuals[29]. As the majority of TB patients in the United Kingdom are immigrants from areas of the world where viral hepatitis is endemic, this strengthens the clinical utility of screening TB patients for viral hepatitis.

The high prevalence of HBV and HCV in our cohort likely reflects the relatively high prevalence of these viruses in their countries of origin. The relevance of our findings is that screening recommendations for HBV for individuals born in high prevalence areas, such as the American Association for the Study of Liver Diseases guidelines on chronic HBV, should be carried out in TB patients and extended to HCV screening.

We propose that screening for hepatitis B and C be considered in TB patients (particularly those with active TB) in multicultural cities of the United Kingdom with high rates of patients originating from areas of the world where HBV and HCV are relatively more highly prevalent. Further studies to identify TB patients at highest risk of HBV/HCV co-infection, to investigate links between active TB and Hepatitis B, and to investigate the health economics of screening are warranted.

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COMMENTS

Background

Tuberculosis (TB) is the leading cause of death from a curable infectious disease. Standard four-drug anti-TB therapy for active disease (isoniazid, rifampicin, pyrazinamide and ethambutol) is associated with a range of significant side effects, London is a multicultural city with a significant number of immigrants from high endemic TB countries, suggesting that the prevalence of viral hepatitis infection may be higher than suspected.

Innovations and breakthroughs

This study aims to assess the prevalence of hepatitis B virus and hepatitis C virus (HCV) infection and association with drug induced liver injury in patients undergoing anti-TB therapy.

Applications

This is the first study from a European centre investigating the prevalence of hepatitis B virus (HBV) and HCV in patients with TB. Chronic HBV and HCV prevalence in TB patients were 9 and 5 times greater than the estimated United Kingdom prevalence respectively, and a significantly greater proportion of patients with active TB had chronic Hepatitis B compared with patients with latent TB infection. In this study there was no association between drug induced liver injury risk and presence of serological markers of HBV/HCV.

Peer-review

This manuscript is a full, complete and very well-written review. There are some minor errors to be amended and some points that could be better addressed to improve its quality.

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ORIGINAL ARTICLE

Prospective Study

Serum proinflammatory cytokines and nutritional status in pediatric chronic liver disease

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Institutional review board statement: This study was reviewed and approved by the Research and Ethics Committee of Hospital de Clinicas de Porto Alegre (protocol n°.11/0078).

Clinical trial registration: The authors of the manuscript entitled "Serum proinflammatory cytokines and nutritional status in pediatric chronic liver disease" declare that this study is registered at the Research and Ethics Committee of Hospital de Clinicas de Porto Alegre, Brazil (IRB00000921). The registration identification number is 11-0078.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

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Abstract

AIM: To evaluate the nutritional status and its association with proinflammatory cytokines in children with chronic liver disease.

METHODS: We performed a cross-sectional study with 43 children and adolescents, aged 0 to 17 years, diagnosed with chronic liver disease. All patients regularly attended the Pediatric Hepatology Unit and were under nutritional follow up. The exclusion criteria were fever from any etiology at the time of enrollment, inborn errors of the metabolism and any chronic illness. The severity of liver disease was assessed by Child-Pugh, Model for End-stage Liver Disease (MELD) and Pediatric End Stage Liver Disease (PELD) scores. Anthropometric parameters were height/age, body mass index/age and triceps skinfold/age according to World Health Organization standards. The cutoff points for nutritional status were risk of malnutrition (Z-score < -1.00) and malnutrition (Z-score < -2.00). Interleukin-1β (IL-1β), IL-6 and tumor necrosis factor-α levels were assessed by commercial ELISA kits. For multivariate analysis, linear regression was applied to assess the association between cytokine levels, disease severity and nutritional status.

RESULTS: The median (25^{th}-75^{th} centile) age of the study population was 60 (17-116)-mo-old, and 53.5% were female. Biliary atresia was the main cause of chronic liver disease (72%). With respect to Child-Pugh score, cirrhotic patients were distributed as follows: 57.1% Child-Pugh A, a mild presentation of the disease, 34.3% Child-Pugh B, a moderate stage of cirrhosis and 8.6% Child-Pugh C, were considered severe cases. PELD and MELD scores were only above the cutoff point in 5 cases. IL-6 values were increased in patients at nutritional risk (34.9%) compared with those who were well-nourished [7.12 (0.58-34.23) pg/mL *vs* 1.63 (0.53-3.43) pg/mL; $P = 0.02$], correlating inversely with triceps skinfold-for-age z-score ($r_s = -0.61$; $P < 0.001$). IL-6 levels were associated with liver disease severity assessed by Child-Pugh score ($P = 0.001$). This association remained significant after adjusting for nutritional status in a linear regression model.

CONCLUSION: High IL-6 levels were found in children with chronic liver disease at nutritional risk. Inflammatory activity may be related to nutritional status deterioration in these patients.

Key words: Cytokines; Interleukin-6; Malnutrition; Cirrhosis; Child

Core tip: Inflammatory activity has been suggested as a component of the pathogenesis of illness-related malnutrition. Several studies have evaluated proinflammatory cytokines in pediatric chronic liver disease, but none have addressed the possible association between these biomarkers and nutritional status. This study showed that the interleukin-6 levels were significantly increased in children and adolescents at nutritional risk. To the best of our knowledge, this is the first study to analyze the relationship between the cytokine levels and nutritional status in children and adolescents with chronic liver disease.

Santetti D, de Albuquerque Wilasco MI, Dornelles CTL, Werlang ICR, Fontella FU, Kieling CO, dos Santos JL, Vieira SMG, Goldani HAS. Serum proinflammatory cytokines and nutritional status in pediatric chronic liver disease. *World J Gastroenterol* 2015; 21(29): 8927-8934 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i29/8927.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i29.8927

INTRODUCTION

Biliary atresia (BA) is an obstructive cholangiopathy that is present at birth or developed during the first two weeks of life[1]. It is the most common cause of chronic liver disease in children and can progress to cirrhosis[2]. The etiology of this disease is still unknown[3]. Late referral to specialized centers is still a problem that can affect patient survival[4]. Alpha-1 antitrypsin deficiency, Alagille syndrome and progressive familial intrahepatic cholestasis (PFIC) are other causes of liver disease in the pediatric population[5]. Complications related to chronic liver disease and cirrhosis include portal hypertension, variceal bleeding, ascites and failure to thrive[6-8].

It is well established that nutritional status is an important factor in the prognosis of chronic liver disease[9]. Growth deficits may influence pre- and post-transplantation mortality[10,11]. The presence of pre-transplant stunting seems to be associated with longer hospital stays and increased costs with hospitalization at the time of transplantation[12]. Early identification of nutritional risk is important so that a multidisciplinary team can implement individualized dietary interventions[13].

Malnutrition in liver disease is related to several conditions, such as reduced caloric intake, anorexia, early satiety, abnormalities in the metabolism of macronutrients, hypermetabolism and increased proinflammatory cytokines[14-16]. In cholestatic patients, there is a significant decrease in the bile acids concentrations in the intestine with consequent malabsorption of lipids and fat-soluble vitamins[17]. This clinical issue remains common, especially in end stage liver disease[18,19].

Recently, inflammatory activity is suggested as a component of the pathogenesis of illness-related malnutrition[20,21]. Moreover, it is possible that proinflammatory cytokines could impair growth and

muscle breakdown through several pathways[20,22]. The inclusion of the assessment of proinflammatory cytokines, such as the concentrations of interleukin-1 beta (IL-1β), IL-6, tumor necrosis factor-alpha (TNF-α) and C-reactive protein (CRP), is currently recommended in routine clinical care[20,23,24].

Many studies have evaluated the cytokine profiles of children with chronic liver disease[25,26], but none have explored the possible association between these biomarkers of inflammation and nutritional status. Given the lack of studies on this relationship, the present study aimed to evaluate the nutritional status of children and adolescents with chronic liver disease, and its association with inflammatory activity, by measuring the proinflammatory cytokines IL-1β, IL-6 and TNF-α.

MATERIALS AND METHODS

Subjects and design

The present cross-sectional study evaluated a total of 43 children and adolescents from 3 mo to 17 years of age with a clinical diagnosis of chronic liver disease. All patients regularly attended the Pediatric Hepatology Unit, Hospital de Clinicas de Porto Alegre, a tertiary reference center for pediatric liver disease and liver transplantation in Southern Brazil. All patients were under regular nutritional follow up and their dietary intake was evaluated as a systematic approach of the multidisciplinary team at our institution.

In our study, cirrhosis was diagnosed by histological and/or standard ultrasonographic and clinical criteria in patients with chronic liver disease. The histological criteria were the presence of nodular formation and fibrosis on liver biopsy. Ultrasonographic findings were the presence of esophageal varices on endoscopy and/or ultrasound, showing heterogeneous echogenicity of the liver and signs of portal hypertension. The clinical criteria were hepatosplenomegaly, ascites, hypoalbuminemia and coagulopathy[27].

The exclusion criteria were as follows: fever of any etiology at the time of enrollment, inborn errors of the metabolism and any chronic illness besides cirrhosis.

Disease severity

The severity of liver disease was assessed according to Child-Pugh score[28]; model for end-stage liver disease (MELD) score[29] for adolescents older than 12 years of age and pediatric end stage liver disease (PELD) score[30] for participants younger than 12 years of age. Scores higher than 15 were considered the cut-off point for liver disease severity.

Anthropometric parameters

The anthropometric parameters used in this study included the following: body mass index-for-age (BMI/A), height-for-age (H/A), mid upper arm circumference-for-age (MUAC/A) and triceps skinfold-for-age (TSF/A), according to World Health Organization (WHO) reference techniques[31,32]. The same trained researcher performed all measurements. The variables were presented as Z-scores. For children under 5 years of age, the WHO Anthro (version 3.2.2) software was applied. Children older than 5 years of age were evaluated using WHO Anthro Plus and anthropometric standards proposed by Frisancho[33] to calculate the MUAC/A and TSF/A. The risk of malnutrition was defined based on Z-score < -1.00 for BMI/A or TSF/A and malnutrition as Z-score < -2.00. For statistical purposes, the two categories, risk of malnutrition and malnutrition, were assembled in a single group called nutritional risk. In patients with ascites, we did not consider values of BMI/A, because this parameter may underestimate the diagnosis of malnutrition due to fluid retention.

Biochemical assays

Blood samples were collected from all patients during the performance of routine tests such as: serum albumin, creatinine, conjugated bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), prothrombin time, international normalized ratio (INR) and CRP. All laboratory tests were executed according to standard operating protocols from the Biochemistry Laboratory of the local institution.

For cytokine assessment (IL-1β, IL-6 and TNF-α), serum (2.0 mL) was immediately separated by centrifugation for 15 min at 3000 rpm and stored at -80 ℃, until analysis. The cytokine concentrations were measured in duplicate using commercial ELISA kits (RD Systems, Inc., Minneapolis, MN, United States) according to the manufacturer's protocol. The minimum detectable levels for cytokines were as follows: 1.0 pg/mL (IL-1β), 0.7 pg/mL (IL-6) and 5.5 pg/mL (TNF-α).

Statistical analysis

Continuous variables were expressed as mean ± SD or median and interquartile range (25^{th}-75^{th} centile). To evaluate the association between categorical variables chi-square test was used. Regarding the correlation between quantitative variables Spearman correlation rank was applied. To compare two groups of quantitative variables we used Mann-Whitney *U* test, and to compare more than two groups, we used Kruskal-Wallis test. The linearity was tested and logarithm adjustment was performed. For multivariate analysis, linear regression was applied to assess the association between the cytokine levels, disease severity and nutritional status. Data were considered statistically significant at $P \leqslant 0.05$.

RESULTS

Subjects

The study population's median (25^{th}-75^{th} centile)

Table 1 Demographic and clinical data of the study population *n* (%)

Variables	Patients (*n* = 43)
Age (yr)	
< 2	13 (30.2)
2-5	9 (20.9)
5-10	11 (25.6)
> 10	10 (23.3)
Female	23 (53.5)
Nutritional status[1]	
Well-nourished	28 (65.1)
Risk of malnutrition	10 (23.3)
Malnutrition	5 (11.6)
Causes of chronic liver disease	
Biliary atresia	31 (72)
Alpha-1 antitrypsin deficiency	6 (14)
Sinusoidal obstruction syndrome	1 (2.3)
Idiopathic chronic liver disease	1 (2.3)
Cirrhosis by cytomegalovirus	1 (2.3)
Cryptogenic cirrhosis	3 (7)
Cholestasis (CB ⩾ 2.0 mg/dL)	12 (27.9)
Albumin (< 3.5 g/L)	12 (27.9)
Portal hypertension	24 (55.8)
Ascites	3 (7)
Hepatomegaly	25 (58.1)
Splenomegaly	32 (74.4)
Cirrhosis	35 (81.4)
Child-Pugh score	
A	20/35 (57.1)
B	12/35 (34.3)
C	3/35 (8.6)
PELD score (> 15)	4/27 (14.8)
MELD score (> 15)	1/8 (12.5)

[1]Parameters for nutritional assessment: triceps skinfold-for-age and body mass index-for-age. CB: Conjugated bilirubin; PELD: Pediatric end stage liver disease; MELD: Model for end stage liver disease.

age was 60 (17-116) mo. BA was the main cause of chronic liver disease (72%). Eight patients had no cirrhosis criteria at the time of enrollment. Of all 35 cirrhotic patients, 24 were diagnosed by liver biopsy. From the remaining 11 without liver biopsy, all had ultrasonographic alterations that were compatible with cirrhosis and portal hypertension (splenomegaly and/or esophageal varices) without portal vein thrombosis.

Regarding Child-Pugh score, cirrhotic patients were distributed as follows: 57.1% Child-Pugh A with a mild presentation of the disease, 34.3% Child-Pugh with a moderate stage of cirrhosis and 8.6% Child-Pugh C were considered severe cases. PELD and MELD scores were only higher than the cutoff point of 15 in 5 cases. Complete demographic and clinical data are shown in Table 1.

Nutritional status

Malnutrition was detected among 11.6% of the children and adolescents with chronic liver disease and 23.3% were considered at risk of malnutrition. The frequency of nutritional risk (risk of malnutrition plus malnutrition) was higher in children younger than 2 years of age, corresponding to 61.5% in this age group (P = 0.037). With respect to linear growth, a frequency of 23.3% of low height-for-age was found.

Table 2 Cytokine levels in children with chronic liver disease at nutritional risk *vs* well-nourished

Cytokine	Nutritional risk	Well-nourished	*P* value[1]
Interleukin-1β (pg/mL)	0.10 (0-0.66)	0.05 (0-0.28)	0.144
Interleukin-6 (pg/mL)	7.12 (0.58-34.23)	1.63 (0.53-3.43)	0.020
TNF-α (pg/mL)	10.74 (8.17-12.35)	6.66 (4.28-11.26)	0.880

[1]Mann-Whitney *U* test. Data are expressed as median (25^{th}-75^{th} centile). TNF-α: Tumor necrosis factor-α.

Table 3 Correlations between the interleukin-6 levels and routine liver function tests

Liver function tests	Median (25^{th}-75^{th})	r_s	*P* value
Aspartate aminotransferase (U/L)	73.50 (49.25-177.00)	0.70	< 0.001
Alanine aminotransferase (U/L)	60.00 (33.75-115.75)	0.49	0.001
Conjugated Bilirubin (mg/dL)	0.7 (0.2-2. 57)	0.72	< 0.001

r_s = Spearman correlation test.

Cytokine assessment

The overall median (25^{th}-75^{th} centile) levels detected for IL-1β, IL-6 and TNF-α were, respectively, 0.07 (0-0.30) pg/mL, 2.2 (0.58-6.8) pg/mL and 8.3 (4.6-11.9) pg/mL. Both IL-6 and TNF-α levels were different between age groups (P = 0.004; P = 0.003).

We found an inverse correlation between IL-6 and TSF/A (r_s = -0.61; P < 0.001), IL-6 and MUAC/A (r_s = -0.51; P = 0.001), and IL-6 and H/A (r_s = -0.34; P = 0.023). IL-1β only correlated inversely with TSF/A (r_s = -0.41; P = 0.006). There was no correlation between the serum TNF-α and TSF/A.

The IL-6 values were significantly increased in patients at nutritional risk (P = 0.02). The cytokine levels in well-nourished children with chronic liver disease compared with those at nutritional risk are presented in Table 2.

Relationship between cytokines and disease severity

A strong correlation was verified between IL-6 and PELD score (r_s = 0.79; P < 0.001) as well as between TNF-α and MELD score (r_s = 0.76; P = 0.017). The IL-6 levels and routine liver function test correlations are shown in Table 3. With respect to albumin, the serum levels correlated inversely with IL-6 levels (r_s = -0.80; P < 0.001) as CRP had a strong positive correlation to this cytokine (r_s = 0.76; P < 0.001).

The IL-1β levels were not associated with liver disease severity assessed through Child-Pugh, PELD and MELD scores in our study population. The TNF-α levels were associated with PELD (P = 0.026) and Child-Pugh scores (P = 0.050).

The IL-6 levels also had a significant association with liver disease severity, which was evaluated by both PELD score (P = 0.014) and Child-Pugh score (P = 0.001). The distribution of this cytokine among

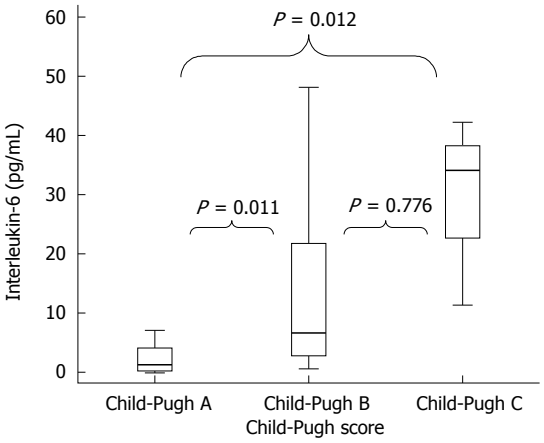

Figure 1 Interleukin-6 levels in children and adolescents with chronic liver disease according to Child-Pugh score.

Child-Pugh's categories A, B and C is shown in Figure 1. In the multivariate model, IL-6 remained associated with the disease severity that was measured by Child-Pugh score and PELD/MELD scores after adjusting for nutritional status (Table 4).

DISCUSSION

This study assessed the relationship between the serum proinflammatory cytokines and nutritional status in children and adolescents with chronic liver disease. We demonstrated that the IL-6 levels were significantly increased in children and adolescents at nutritional risk enrolled in our study. To the best of our knowledge, this is the first study to analyze the relationship between the cytokine levels and nutritional status in children and adolescents with chronic liver disease.

Our results agreed with previous findings that suggested that the serum concentrations of IL-6 could be used to identify patients at risk of nutritional deterioration[34]. Illness-related malnutrition appears to display an association with inflammatory components through increased resting energy expenditure, decreased calorie consumption, anorexia, nutrient loss, altered nutrient utilization and malabsorption[20]. This concept seems to be appropriate for children and adolescents with chronic liver disease who have an intense protein catabolism, changes in body composition and muscle loss.

Increased resting energy expenditure could be related to weight and growth impairment in pediatric chronic liver disease, which may be approximately 30% higher compared with a healthy child[35]. This hypermetabolic state could be due to inflammation by the presence of ascending cholangitis, ascites and disordered substrate and energy uptake[15]. Additionally, altered intestinal permeability with consequent endotoxemia is directly linked to increased cytokine production observed in cirrhosis. It is suggested

Table 4 Multiple linear regression models for interleukin-6 levels adjusted for liver disease severity and nutritional status

Models[1]	β	*P* value
Child-Pugh score[2]	0.581	< 0.001
PELD/MELD score[3]	0.535	0.001

[1]Logarithmic transformation was applied; [2]Adjusted for nutritional status. R^2 = 37.8%; [3]Adjusted for nutritional status. R^2 = 30.6%. PELD: Pediatric end stage liver disease; MELD: Model for end stage liver disease.

that malnourished cirrhotic patients have increased intestinal permeability compared with those who are well-nourished[36], which could explain the higher levels of IL-6 found in our patients at nutritional risk. However, in cirrhotic adults, increased systemic levels of IL-6 did not correlate with body mass index[37]. We can assume that there are still conflicting data related to the possible relationship between malnutrition and proinflammatory cytokines production, especially in chronic liver disease.

The pathophysiology of malnutrition is thought to be the combined influence of undernutrition and inflammatory activity in the body composition[38]. It is well established that the presence of inflammatory activity induces peripheral loss of lean body mass[24]. In our study, we found a strong inverse correlation between the IL-6 levels and TSF/A Z-score, a parameter of body composition. This finding agrees with a possible link between the loss of lean body mass and inflammatory activity. However, these associations were not confirmed for IL-1β and TNF-α, which is in agreement with a study on cystic fibrosis patients[39]. Anthropometric parameters, such as TSF/A, are important tools for the nutritional assessment of cirrhotic patients, especially at advanced stages of the disease. It has been reported that there is a correlation between the liver disease severity, estimated by liver function tests, and impaired nutritional status, measured by anthropometric markers[40].

Concerning nutritional assessment tools, it is well known that serum albumin shows low sensitivity and specificity as a marker of nutritional status, especially in patients with liver disease. Furthermore, it is assumed that albumin could be indicative of the presence of inflammation[41]. In agreement with this theoretical assumption, we found a strong inverse correlation between the IL-6 levels and albumin. With respect to the relationship between CRP and IL-6, there was a positive correlation in our study once this cytokine stimulates the release of CRP by the liver during the acute phase of inflammation. A recent study reported that this acute-phase protein could also be used as a tool to predict short-term mortality in severely hospitalized cirrhotic patients[42].

Recent studies have suggested that IL-6 plays an important role in the pathogenesis of several diseases[43,44]. Focusing on liver disease patients, there seems to be an imbalance between proinflammatory

and anti-inflammatory cytokines in cirrhosis. The activated Kupffer cells of the liver are involved in cytokine secretion. A study comparing children with BA and intra-hepatic cholestatic diseases have reported higher values of IL-6 in the BA group, indicating the presence of chronic inflammation. Nevertheless, there was no mention of the patient's nutritional status. The authors concluded that IL-6 could contribute to determining the disease severity[45]. As for these studies, we found an association between the liver disease severity, assessed by Child-Pugh score, and increased IL-6 levels. Moreover, we also found a positive correlation between IL-6 and routine liver function tests, such as AST, ALT and CB.

Increased lipid oxidation and decreased glucose uptake were demonstrated by indirect calorimetry in cirrhotic patients, and this metabolic abnormality is correlated with the disease severity and circulating levels of TNF-α[46]. In our study, the TNF-α levels were also associated with liver disease severity, but they were not associated with the nutritional status assessed by anthropometric parameters. The IL-1β levels were not significantly related to the outcomes in our study. However, it is well established that proinflammatory cytokines can act through stimulating one another's production[47]. We can speculate that this may have occurred in our study, because IL-1β could be somehow stimulating IL-6 and TNF-α production.

The limitation of the study may be due to the small number of severe liver disease patients (Child-Pugh C) because all patients were enrolled in the outpatient clinic. Moreover, the study design (cross sectional) did not allow for medium or long-term follow up to assess the nutritional and clinical outcomes. The strength of this study is that we found the relationship between high IL-6 levels in children and adolescents with chronic liver disease at nutritional risk. However, we could not determine the exact cause of increased IL-6 levels, which could be from either malnutrition or chronic liver disease or both.

We reported a relationship between high IL-6 levels and nutritional status in children and adolescents with chronic liver disease. Our findings suggest that inflammatory activity appears to be part of the evolution of pediatric chronic liver disease. Extensive knowledge of this inflammatory panorama is of main importance in our field, enabling the creation of new approaches to nutritional support. Further research is required to evaluate the effect of dietary intervention on inflammatory response, in children and adolescents with chronic liver disease.

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COMMENTS

Background

Malnutrition in liver disease is frequently related to decreased caloric intake, early satiety, and abnormalities in the metabolism of macronutrients. Additionally, it has also been hypothesized that malnutrition might be related to inflammatory activity in patients with chronic diseases.

Research frontiers

This is essentially a clinical study that assessed the relationship between the cytokine levels and nutritional status in children and adolescents with chronic liver disease.

Innovations and breakthroughs

Several studies evaluated the cytokine profiles of children with chronic liver diseases, but none addressed the possible association between these biomarkers of inflammation and nutritional status. This is the first study to analyze the relationship between the cytokine levels and nutritional status in children and adolescents with chronic liver disease.

Applications

Understanding the role of proinflammatory cytokines, such as IL-6, in pediatric chronic liver disease, might be a helpful tool for assessing the illness-related malnutrition presented by these patients.

Terminology

Cytokines are inflammatory mediators produced by T cells. The circulating levels of these biomarkers are identified in the presence of overproduction with consequent impact on homeostasis. Proinflammatory cytokines, such as Interleukin-1β (IL-1β), IL-6 and tumor necrosis factor-α, may be associated with illness-related malnutrition.

Peer-review

This manuscript highlights the association between the IL-6 levels and nutritional status deterioration. Their findings suggest that inflammatory activity appears to be part of the evolution of pediatric chronic liver disease. Extensive knowledge of this inflammatory panorama is of primary importance in their field, enabling the creation of new approaches to nutritional support.

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ORIGINAL ARTICLE

Prospective Study

Interferon-λ polymorphisms and response to pegylated interferon in Iranian hepatitis C patients

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Author contributions: Keshvari M and Pouryasin A designed the research; All patients were evaluated and treated by Keshvari M; Sharafi H and Pouryasin A performed the molecular assessments; Hemmati K and Mohammadzadehparjikolaei F extracted the data from the patients' documents and completed the side effect questionnaires by interviewing the patients; Sharafi H performed the statistical analysis; and Haj-sheykholeslami A wrote the article and the final draft was reviewed by Keshvari M.

Supported by Pooyesh Darou which is the local manufacturer of pegylated interferon alpha-2a in Iran (Pegaferon).

Institutional review board statement: This study was approved by the Ethics Committee of the Iranian Blood Transfusion Organization. The study protocol conformed to the ethical guidelines of the 1975 declaration of Helsinki.

Informed consent statement: Informed consent was obtained from all patients participating in the study.

Conflict-of-interest statement: This study was financially supported by Pooyesh Darou, which is the local manufacturer of pegylated interferon alpha-2a in Iran (Pegaferon®). The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Data sharing statement: Dataset is available from the corresponding author at m.keshvari@ibto.ir.

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Abstract

AIM: To evaluate the efficacy of pegylated interferon in Iranian chronic hepatitis C patients in relation to interferon-λ (IFNL) polymorphisms.

METHODS: This study enrolled patients with chronic hepatitis C referred to the Tehran Blood Transfusion Hepatitis Clinic in 2011. Patients were included in the study if they had no concomitant hepatic illness, were negative for human immunodeficiency virus antibodies, and had no prior history of treatment with any type of

pegylated interferon. Patients were treated with 180 μg pegylated interferon alpha-2a (Pegaferon®) weekly and 800-1200 mg ribavirin daily for 24 or 48 wk depending on weight and hepatitis C virus (HCV) genotype. Blood samples were collected from patients to obtain DNA for determination of *IFNL* rs12979860 and rs8099917 polymorphisms. The virologic response in patients was then evaluated and compared between the different *IFNL* genotypes.

RESULTS: A total of 152 patients with a mean age of 41.9 ± 10.0 years were included in the study, of which 141/152 were men (92.8%). The most frequent HCV genotype was type-1, infecting 93/152 (61.2%) patients. Sustained virologic response (SVR) was achieved in 81.9% of patients with HCV genotype-1 and 91.1% of patients with HCV genotype-3. Treatment success was achieved in 91.2% (52/57) of patients with the *IFNL* rs12979860 CC genotype and 82.1% (78/95) in those with other genotypes. Similar treatment response rates were also observed in patients with rs8099917 TT (39/45; 86.7%) and non-TT (61/68; 89.7%) genotypes. Univariate analyses identified the following factors which influenced treatment response for inclusion in a multivariate analysis: age, HCV RNA level, stage of liver fibrosis, rs12979860 CC genotype, and aspartate transaminase level. A logistic regression analysis revealed that only the rs12979860 CC genotype was significantly associated with achievement of SVR (OR = 6.2; 95%CI: 1.2-31.9; P = 0.03).

CONCLUSION: The rs12979860 CC genotype was associated with SVR in patients receiving pegylated interferon plus ribavirin, however, the SVR rate in other rs12979860 genotypes was also relatively high.

Key words: Chronic hepatitis C; Pegylated interferon; rs12979860; rs8099917; Sustained virologic response

Core tip: Chronic hepatitis C-infected Iranian patients treated with pegylated interferon and ribavirin showed relatively high rates of sustained virologic response. Treatment success was not influenced by hepatitis C virus genotype. However, a comparison of treatment success related to *IFNL* polymorphisms (also known as *IL28B* polymorphisms) using a logistic regression analysis revealed that the interferon-λ rs12979860 CC genotype was significantly associated with achieving a sustained virologic response.

Haj-sheykholeslami A, Keshvari M, Sharafi H, Pouryasin A, Hemmati K, Mohammadzadehparjikolaei F. Interferon-λ polymorphisms and response to pegylated interferon in Iranian hepatitis C patients. *World J Gastroenterol* 2015; 21(29): 8935-8942 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i29/8935.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i29.8935

INTRODUCTION

Chronic infection with the hepatitis C virus (HCV) is a serious condition that can lead to cirrhosis or hepatocellular carcinoma. An estimated 150 million people worldwide are chronically infected with HCV, which is responsible for 350000 liver-related deaths annually[1]. In Iran, the prevalence of chronic HCV infection is approximately 0.5%[2]. With the advent of new potent drugs such as direct acting oral agents, recommendations for chronic hepatitis C treatment in adults is rapidly changing. Although these new agents are more effective and have fewer side effects they are offered at very high prices, making pegylated interferon (Peg-IFN) plus ribavirin the only affordable treatment option for a large group of patients, especially those in developing countries[3]. Until recently the Peg-IFN regimen was the mainstay of chronic hepatitis C treatment, and resulted in a sustained virologic response (SVR) in 33%-80% of patients[4,5]. However, data regarding the response rate of Iranian patients to Peg-IFN is limited, with the reported success rates ranging from 50%[6] to 61%[7]; although these studies were not restricted to the evaluation of treatment-naïve patients.

Treatment outcome can be affected by various factors, including single nucleotide polymorphisms (SNPs) of interferon-λ (*IFNL*) (located near the *IL28B* gene and thus also known as *IL28B* polymorphisms) such as rs12979860 and rs8099917[8]. These SNPs are also associated with spontaneous clearance of HCV[9,10]. Studies have shown that rs12979860 "CC" and rs8099917 "TT" genotypes are significantly associated with preferred treatment outcome[11,12]. Thus, to further investigate the role of these SNPs, the success of Peg-IFN-alpha-2a plus ribavirin treatment in Iranian patients with chronic HCV infection was evaluated with respect to host genetics and HCV genotypes.

MATERIALS AND METHODS

Study population

Adult patients with chronic HCV infection (defined as the presence of HCV RNA in serum for > 6 mo) referred to the Tehran Blood Transfusion Hepatitis Clinic between March 2011 and August 2013 were enrolled in the study. Patients with no concomitant hepatic illness, negative results for human immunodeficiency virus antibody and hepatitis B surface antigen, and no history of prior antiviral therapy for HCV infection were included in the study. A majority of the patients underwent percutaneous liver biopsy, for which specimens were evaluated according to the modified Knodell score grading and staging system by Ishak *et al*[13].

Treatment regimen

Patients received weekly subcutaneous injections of Peg-IFN-alpha-2a (180 μg/mL Pegaferon®; Pooyesh Darou, Tehran, Iran) and daily oral ribavirin (Ribabiovir®;

Bakhtar Bioshimi, Kermanshah, Iran): 800 mg for HCV genotype-3 and 1000-1200 mg according to the patient's weight (< or ⩾ 75 kg) for HCV genotype-1.

Treatment duration was 24 wk for those infected with HCV genotype-3, or 48 wk for infection with HCV genotype-1, but could be shortened or extended depending on the patient's response and compliance.

The patients were closely monitored throughout the treatment course, with monthly complete blood cell count and liver enzyme tests, and HCV RNA level assessment before treatment, at weeks 4, 12, 24, at the end of treatment, and 24 wk after treatment completion. Informed consent was obtained from all participating patients. The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki, and was approved by the Ethics Committee of the Iranian Blood Transfusion Organization.

Laboratory assessments

Quantitative assessment of HCV RNA was performed using the COBAS TaqMan HCV Test v2.0 (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions and a detection limit of 10 IU/mL. HCV genotyping was performed using HCV genotype-specific primers[14].

The *IFNL* SNPs were assessed using the PCR-restriction fragment length polymorphism method[15]. Briefly, genomic DNA was extracted from patients' peripheral blood using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The PCR was performed using Accupower PCR PreMix (Bioneer Corp., Daejeon, South Korea) with the following conditions: 94 ℃ for 5 min, 35 cycles of 94 ℃ for 20 s, 66 ℃ for 20 s, and 72 ℃ for 20 s, followed by 72 ℃ for 5 min. Primers used for the reaction included: rs12979860, 5'-GCGGAAGGAGCAGTTGCGCT-3' and 5'-GGGGCTTTGCTGGGGGAGTG-3'; or rs8099917, 5'-CCCACTTCTGGAACAAATCGTCCC-3' and 5'-TCTCCTCCCCAAGTCAGGCAACC-3'. The PCR amplicons were then digested for ⩾ 1 h with 10 U of restriction endonuclease: *Bst*UI for rs12979860 or *Bsr*DI for rs8099917 (Fermentas of Thermo Fisher Scientific, Waltham, MA, United States). The digested PCR products were separated on 3% agarose gels revealing the following sized fragments: rs12979860, 196 and 45 bp for the CC genotype, 241, 196, and 45 bp for the CT genotype, or 241 bp for the TT genotype; rs8099917, 552 bp for the TT genotype, 552, 322 and 230 bp for the GT genotype, and 322 and 230 bp for the GG genotype.

Assessment of treatment response

Therapeutic responses were categorized as: rapid virologic response (RVR; HCV RNA undetectable after 4 wk of treatment), early virologic response (EVR; HCV RNA undetectable or ⩾ 2 log decreased at 12th wk of treatment), or sustained virologic response (SVR; HCV RNA undetectable at 6 mo after the end of treatment). Achievement of SVR was considered as treatment success, and thus used to describe patients as responders or non-responders. HCV breakthrough was used to describe cases where HCV RNA was detected during treatment after a previous period where it was undetectable, and resistant infections were cases where HCV RNA was detectable at all times. If HCV RNA was undetectable at the end of therapy, but then detected 6 mo later, this was considered a relapse[16].

Statistical analysis

Statistical analyses were performed using SPSS v16.0 software (SPSS Inc., Chicago, IL, United States). The association of each nominal variable with SVR achievement was evaluated with cross tabulation and χ^2 testing. Independent sample Student's *t* tests were used for continuous variables with a normal distribution, otherwise the Mann-Whitney *U* test was used. All baseline variables that had a $P < 0.2$ in univariate tests were entered into a logistic regression model, and $P < 0.05$ was considered statistically significant. The statistical methods used in this study were reviewed by Maryam Sharafkhah, MS, of the Biostatistics in Digestive Diseases Research Institute.

RESULTS

Patient characteristics

A total of 152 patients (141 men and 11 women; mean age 41.9 ± 10.0 years) with chronic HCV infection were included in this study. The mean HCV RNA level was 3920258 ± 6856760 IU/mL (interquartile range: 4420288 IU/mL). A larger proportion of patients (93/152; 61.2%) were infected with HCV genotype-1 (1a: *n* = 86; 1b: *n* = 7), and the remaining patients were infected with HCV genotype-3a (*n* = 56), a mixed genotype-1a/3a (*n* = 2), or mixed genotype-1a/4 (*n* = 1).

The frequencies of *IFNL* rs12979860 CC, CT, and TT genotypes were 37.5%, 47.3%, and 13.2%, respectively, and were 60.2%, 37.2%, and 2.7% for rs8099917 TT, GT, and GG genotypes, respectively. There were no significant differences in rs12979860 and rs8099917 subtype frequencies with respect to HCV genotype.

Liver biopsies were performed in 99/152 (65.1%) patients, indicating stage 0 (*n* = 8), stage 1 (*n* = 23), stage 2 (*n* = 26), stage 3 (*n* = 27), or stage 4 (*n* = 4) fibrosis, or cirrhosis (*n* = 11).

Treatment response

Treatment duration was reduced to < 48 wk for 24/94 (25.5%) patients with HCV genotype-1 infection (15 with shortened treatment course, two as a result of non-compliance, and seven following treatment failure), and to < 24 wk in one non-compliant HCV genotype-3 subject. Treatment was prolonged up to

Table 1 Treatment outcomes *n* (%)

Hepatitis C virus genotype	Virologic response				Resistant	Breakthrough	Relapse
	Rapid	Early	End of treatment	Sustained			
Type-1[1] (*n* = 94)	42 (44.7)	86 (91.5)	85 (90.4)	77 (81.9)	6 (6.4)	3 (3.2)	8 (8.5)
Type-3 (*n* = 56)	47 (83.9)[b]	49 (89.1)[2]	56 (100)	51 (91.1)	0 (0)	0 (0)	5 (8.9)
Mixed type-1 and -3 (*n* = 2)	1 (50.0)	2 (100)	2 (100)	2 (100)	0 (0)	0 (0)	0 (0)
Total (*n* = 152)	90 (59.2)	137 (90.7)[2]	143 (94.1)	130 (85.5)	6 (3.9)	3 (2.0)	13 (8.6)

[1]Includes one patient with mixed hepatitis C virus genotype-1a/4; [2]Data from one patient was missed; [b]$P < 0.01$ *vs* type-1.

Table 2 Baseline variables according to treatment response

Variable	All subjects		HCV genotype-1		HCV genotype-3	
	R (*n* = 130)	NR (*n* = 22)	R (*n* = 77)	NR (*n* = 17)	R (*n* = 51)	NR (*n* = 5)
Age (yr)	40.9 ± 9.4[a]	47.7 ± 11.5	41.7 ± 9.0	47.9 ± 11.9	40.0 ± 10.1	46.8 ± 8.9
HCV RNA (× 10^5 IU/mL)	35.6 ± 55[a]	60.1 ± 99.5	37.9 ± 48.6	70.1 ± 111.6	32.4 ± 64.9	26.3 ± 16.8
Liver fibrosis stage	2.2 ± 1.5	3.0 ± 1.7	2.3 ± 1.5	3.0 ± 1.8	1.8 ± 1.3	3.0 ± 0.0
ALT (IU/L)	71.9 ± 57.7	100.9 ± 108.7	74.2 ± 63.9	77.9 ± 63.7	70.1 ± 47.9	198.7 ± 203.6
AST (IU/L)	44.5 ± 26.4	71.2 ± 66.8	44.9 ± 28.7	58.5 ± 47.6	44.4 ± 23.3	125.2 ± 113.5
BMI (kg/m^2)	26.9 ± 4.0	25.8 ± 3.4	27.2 ± 3.5a	25.1 ± 3.0	26.2 ± 4.6	29.5 ± 3.7

Data are presented as mean ± SD; [a]$P < 0.05$ *vs* NR. ALT: Alanine transaminase; AST: Aspartate transaminase; BMI: Body mass index; HCV: Hepatitis C virus; NR: Non-responder; R: Responder (achieved sustained virologic response).

72 wk in 14/94 (14.9%) patients with HCV genotype-1 infection because of cirrhosis or the continued presence of HCV RNA at 12 wk. Similarly, 10 patients with HCV genotype-3 infection required an extended 48-wk therapy course. Treatment course was withdrawn in 1 subject due to attempted suicide. The 11 patients with cirrhosis did not show signs of ascites or thrombocytopenia, and treatment was therefore administered.

The treatment outcomes for all patients and according to HCV genotypes are shown in Table 1. Although the rates of EVR and SVR did not differ between the HCV genotype-1 and genotype-3 groups, the rate of RVR was significantly higher in patients with HCV genotype-3 than in patients with HCV genotype-1 ($P < 0.01$). Furthermore, 44/62 (71.0%) patients who did not demonstrate RVR and 7/14 (50.0%) patients who did not show EVR eventually achieved SVR. Therefore, overall, RVR and EVR were significantly related to treatment success ($P < 0.01$).

Predictors of treatment response

The age, baseline HCV RNA level, liver fibrosis stage, serum alanine and aspartate transaminase levels, and body mass index were compared among treatment responders and non-responders within the HCV genotype groups (Table 2). Patients with mixed HCV genotypes were excluded from analyses due to the small sample size. The baseline characteristics did not differ between HCV genotype groups, although responders were significantly younger ($P < 0.01$), and had lower HCV RNA levels ($P = 0.04$) than non-responders. These differences were not significant within the HCV genotype subgroups. In contrast, body mass index did not differ within the entire cohort with regard to treatment response, however, responders with HCV genotype-1 infection had a significantly higher body mass index than non-responders ($P = 0.03$).

Table 3 shows the prevalence of rs12979860 and rs8099917 genotypes with respect to treatment response. Among subjects with rs12979860 CC genotype, 91.2% (52/57) responded successfully to treatment, whereas 82.1% (78/95) of patients with a non-CC genotype responded. Treatment success occurred in 86.7% (39/45) of patients with the rs8099917 TT genotype, and in 89.7% (61/68) of those with a non-TT genotype. Univariate analyses revealed that there were no differences in the prevalence of the *IFNL* genotypes between responders and non-responders for either polymorphism. However, among patients with HCV genotype-1 infection, the rs12979860 CC genotype was significantly related to treatment success (94.1% *vs* 75.0%; $P = 0.02$). This was not observed in the HCV genotype-3 group.

Patients with the rs12979860 CC genotype had significantly higher HCV RNA levels compared to those with a non-CC genotype ($60.9 \times 10^5 \pm 81.8 \times 10^5$ IU/mL *vs* $26.4 \times 10^5 \pm 45.7 \times 10^5$ IU/mL, $P = 0.01$). Further analyses also revealed that RNA levels of HCV

Table 3 Prevalence of single nucleotide polymorphisms *n* (%)

Hepatitis C virus genotype	rs12979860 genotype		rs8099917 genotype[1]	
	CC	Non-CC	TT	Non-TT
Type-1				
Responders	32 (41.6)	45 (58.4)	32 (59.3)	22 (40.7)
Non-responders	2 (11.8)	15 (88.2)	3 (33.3)	6 (66.7)
Type-3				
Responders	20 (39.2)	31 (60.8)	28 (63.6)	16 (36.4)
Non-responders	3 (60.0)	2 (40.0)	4 (100)	0 (0)
Mixed type-1 and -3				
Responders	0 (0)	2 (100)	1 (50.0)	1 (50.0)
Non-responders	0 (0)	0 (0)	0 (0)	0 (0)
Total				
Responders	52 (40.0)	78 (60.0)	39 (39.0)	61 (61.0)
Non-responders	5 (22.7)	17 (77.3)	7 (53.8)	6 (46.2)

[1]Data missing for 39 patients.

genotype-1 were significantly higher in patients with rs12979860 CC *vs* other genotypes ($71.6 \times 10^5 \pm 88.1 \times 10^5$ IU/mL *vs* $28 \times 10^5 \pm 40.2 \times 10^5$ IU/mL, $P < 0.01$). However, this was not the case for patients in the HCV genotype-3 group. In addition rs12979860 CC and rs8099917 TT genotypes were significantly related to achievement of RVR (both $P < 0.05$), but not EVR. RVR was also significantly associated with rs12979860 CC and rs8099917 TT genotypes in patients infected with HCV genotype-1 ($P = 0.01$).

Logistic regression analysis of treatment success was conducted according to age, categorical HCV RNA level, stage of liver fibrosis, rs12979860 CC genotype, and aspartate transaminase level (variables identified by $P < 0.2$ in univariate analyses). The results showed that only rs12979860 CC genotype was a predictor of SVR achievement ($P = 0.03$). Table 4 shows the results of multivariate analysis.

Table 4 Predictive factors of treatment success

Baseline variable	*P* value	Odds ratio	95%CI
Age	0.18	0.96	0.91-1.02
AST	0.55	0.99	0.98-1.01
HCV RNA level (categorical)	0.30	0.53	0.16-1.75
Stage of liver fibrosis	0.22	0.77	0.50-1.17
rs12979860 CC genotype	0.03[1]	6.23	1.22-31.88

[1]Significant association with SVR achievement. AST: Aspartate transaminase; HCV: Hepatitis C virus; SVR: Sustained virologic response.

DISCUSSION

A wide range of response rates has been reported for Peg-IFN and ribavirin treatment of chronic HCV infection, reflecting the various settings and patient selections. For example, these values range from 7% in patients with HCV genotype-1 and high viral load[17] to 80% in patients with HCV genotype-2 and -3[5,18]. Although recently more effective treatments for chronic hepatitis C have been introduced, the high cost of these treatments has made the Peg-IFN plus ribavirin regimen the only affordable treatment option in many developing countries[3]. Jabbari *et al*[19] reported a high success rate with this regimen in Iranian patients with HCV genotype-3 compared to genotype-1 (95% *vs* 67%), and another Iranian study reported an 83.8% success rate in patients with HCV genotype-3 and 72.6% in those with HCV genotype-1[20]. Moreover, Alavi Moghaddam *et al*[21] reported an excellent treatment success rate of 95.6% in 45 younger hemophiliac patients (mean age 30.4 ± 12.6 years) with HCV genotypes-1 and -3. The results from these Iranian studies are in line with our results presented herein. Studies on the success rate of this treatment regimen in neighboring countries are rare and comparing the results of the existing ones is also difficult due to different HCV genotype distributions. A study from Turkey reported SVR rates of 48.6% and 35.1% in a group of patients with HCV genotype-1 treated with Peg-IFN-alpha-2a or Peg-IFN-alpha-2b, respectively[22].

A report by Muir *et al*[23] found that responses to Peg-IFN varied among ethnicities regardless of the HCV genotype, with the lowest rates (22%) in African Americans, and the highest (59%) in Asian Americans. Another study reported an SVR rate of 76% in Asian patients *vs* 36% in Caucasians[24]. Thus, ethnic differences may explain the higher success rates in the Iranian patients reported here and elsewhere[21].

Another interesting finding in our study was the same treatment success rate in HCV genotype- 1 and -3 patients. Lin *et al*[25] showed that patients younger than 40 with HCV genotype-1 had a treatment response similar to HCV genotype-2 infection. Moreover, a study by Gheorghe *et al*[26] showed that patients with HCV genotype-1 and mild hepatitis had a high rate of SVR similar to those with other HCV genotypes, young age and low level of viremia and significant hepatocytolisis were found to be independent predictors of SVR. These findings can explain the similar SVR achievement rates between the 2 HCV genotype groups in our study regardless of IFNL polymorphism. It is also possible that apart from ethnic differences, the relatively younger age of the participants and their high compliance (3.6% dropout rate) in our study accompanied by the fact that we only included treatment-naïve patients contributed to the observed high treatment success rate.

The identification of rs12979860 and rs8099917 genotypes has been recommended prior to treatment of patients infected with HCV genotype-1 or -4 due to the possibility of a more favorable outcome[8]. Although a meta-analysis performed by Belgian scientists demonstrated that favorable *IL28B* genotypes are associated with higher RVR and SVR rates in HCV genotype-2 and -3 patients, but as their impact on SVR was slim, they did not recommend *IL28B* genotyping before therapeutic interventions in these patients[11].

The findings reported herein indicate that only the rs12979860 CC genotype significantly affects SVR, which is consistent with another previous Iranian study on 48 patients infected with HCV genotype-1, where the SVR rate was higher in those with the rs12979860 CC genotype in comparison to those with rs12979860 TT genotype[27]. Nevertheless, in the present study, both rs12979860 and rs8099917 were related to RVR, but not EVR achievement, regardless of HCV genotype. The distributions of rs12979860 and rs8099917 SNPs observed in this study were concordant with previous reports[28,29].

As we only included 152 subjects in our study and had a high treatment success rate, only 22 patients failed to achieve SVR, therefore a larger sample size with more subjects in the non-responder group may have confirmed these results with more certainty. Although in this study reaching RVR and EVR were both significantly related to treatment success, SVR was still achieved in more than half of the patients that initially had failed to reach RVR or EVR (71% and 50%, respectively).

The results of the present study also show that patients with the rs12979860 CC genotype had more than two times the HCV RNA level than non-CC genotypes, which has been reported for various HCV genotypes throughout the various courses of infection (acute, early chronic, and chronic)[30-33]. Bucci *et al*[34] reported similar results in 201 HCV genotype-3 patients from the United Kingdom, this study showed that favorable IL28B polymorphisms (rs8099917) are associated with a marked increase in baseline viral load and RVR achievement, but not SVR. Domagalski *et al*[32] also confirmed the association between favorable rs12979860 CC genotype and higher baseline viral loads in patients with HCV genotype 1 and 4. Abe *et al*[35] reported more severe inflammation and fibrosis in the homozygous bearers of major IL28B alleles questioning their benefit outside the treatment context. It is suggested that different IL28B polymorphisms induce different cytokine profiles that can cause different inflammatory or biochemical results in the course of chronic HCV infection. This apparent paradox between higher baseline HCV RNA level, which has been linked to treatment failure in many studies[36,37], and higher response rate in patients with the rs12979860 CC genotype requires further investigation.

More large scale multi-centric studies are needed to reliably evaluate the response rate of patients in different ethnical or geographical parts of the world and to identify the responsible factors for high success rates in specific populations to improve the treatment outcome for all patients with chronic hepatitis C.

In conclusion, this study shows that the SVR achievement rate is high in Iranian treatment-naïve patients regardless of HCV genotype. Although the rs12979860 CC genotype showed a strong relationship with SVR achievement, the comparatively high level of SVR achievement with Peg-IFN and ribavirin in patients with other rs12979860 genotypes indicates that the Iranian patient's *IFNL* polymorphism should not alter treatment decisions.

COMMENTS

Background

Chronic hepatitis C virus (HCV) infection is a major public health problem that can result in cirrhosis and lead to hepatocellular carcinoma or even death. For the past decade, Pegylated interferon (Peg-IFN) has been the primary therapeutic intervention for chronic HCV infection, although its efficacy is influenced by many factors, including single nucleotide polymorphisms in or near the *IL28B* gene.

Research frontiers

Many studies have reported wide ranging efficacies for Peg-IFN and ribavirin in the treatment of chronic HCV infection. Although patient age, viral load, and ethnicity can substantially affect treatment success, the role of host genetic factors, such as single nucleotide polymorphisms in relevant genes, are becoming more apparent. Thus, further understanding of the influence of these factors will help improve patient outcome.

Innovations and breakthroughs

The high response rate to Peg-IFN and ribavirin is an important finding of this study, and may support the decision to accept treatment by many patients with chronic HCV infection, particularly those in developing countries. The high response rate of Iranian patients to Peg-IFN and ribavirin in this study highlights that the conventional dual therapy can still be considered the first-line treatment for these patients.

Applications

The results of this study show that the rs12979860 CC genotype is a strong predictor of treatment response. Thus, patients with this rs12979860 genotype can achieve greater benefit from Peg-IFN and ribavirin treatment.

Terminology

Interferon-λ is a cytokine that is involved in host defense against viral infections, such as hepatitis C. Single nucleotide polymorphisms are genetic variations within the DNA that are common within a population.

Peer-review

The authors carried out a cross-sectional multivariate analysis to describe positive predictive factors for response to HCV treatment with Peg-IFN alpha-2a plus ribavirin in a naïve Iranian cohort. Special attention is given to *IL28B* polymorphisms as a target to predict sustained viral response. They observed a similar finding to previous reports showing a relationship between the *IFNL* rs12979860 CC genotype and a higher sustained virologic response probability. The patient cohort demonstrated a high sustained virologic response rate overall, which could be related to ethnic factors that render the Iranian population more sensitive to Peg-IFN plus ribavirin treatment.

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ORIGINAL ARTICLE

Prospective Study

Esophagojejunostomy after laparoscopic total gastrectomy by OrVil™ or hemi-double stapling technique

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Conflict-of-interest statement: All authors declare that they have no conflict of interest.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at guanwenxiansci@126.com. No additional data are available.

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Abstract

AIM: To investigate the feasibility, advantages and disadvantages of two types of anvil insertion techniques for esophagojejunostomy after laparoscopic total gastrectomy.

METHODS: This was an open-label prospective cohort study. Laparoscopy-assisted radical total gastrectomy with D2 lymph node dissection was performed in 84 patients with primary non-metastatic gastric cancer confirmed by pre-operative histological examination. Overweight patients were excluded, as well as patients with peritoneal dissemination and invasion of adjacent organs. After total gastrectomy, all patients were randomized into two groups. Patients in Group Ⅰ underwent esophagojejunostomy using a transorally-inserted anvil (OrVil™), while patients in Group Ⅱ underwent esophagojejunostomy using the hemi-double stapling technique (HDST). Both types of esophagojejunostomy were performed under laparoscopy. Patients' baseline characteristics, preoperative characteristics, perioperative characteristics, short-term postoperative outcomes and operation cost were compared

between the two groups. The primary endpoint was evaluation of the surgical outcome (operating time, time of digestive tract reconstruction and time of anvil insertion) and the medical cost of each operation (operation cost and total cost of hospitalization). The secondary endpoints were time to solid diet, post-surgical hospitalization time, time to defecation, time to ambulation and intra-operative blood loss. In addition, complications were assessed and compared.

RESULTS: Laparoscopic total gastrectomy and esophagojejunostomy were successfully performed in all 84 patients, without conversion to laparotomy. There were no significant differences in the operative time and time for total gastrectomy between the two groups (287.8 ± 38.4 min *vs* 271.8 ± 46.1 min, P = 0.09, and 147.7 ± 31.6 min *vs* 159.8 ± 33.8 min, P = 0.09, respectively). The time for digestive tract reconstruction and for anvil insertion were significantly decreased in Group Ⅱ compared with Group I (47.8 ± 12.1 min *vs* 55.4 ± 15.7 min, P = 0.01, and 12.6 ± 4.7 min *vs* 18.7 ± 7.5 min, P = 0.001, respectively). Intra-operative blood loss (96.4 ± 32.7 mL *vs* 88.2 ± 36.9 mL, P = 0.28), time to defecation (3.5 ± 0.9 d *vs* 3.2 ± 1.1 d, P = 0.12), time to ambulation (3.9 ± 0.7 d *vs* 3.6 ± 1.1 d, P = 0.12), time to solid diet (7.6 ± 1.4 d *vs* 8.0 ± 2.7 d, P = 0.31) and total hospitalization (10.6 ± 2.6 d *vs* 10.8 ± 3.5 d, P = 0.80) were similar between the two groups. In addition, the total costs of hospitalization were similar between the two groups (73848.7 ± 11781.0 RMB *vs* 70870.3 ± 14003.5 RMB, P = 0.296), but operation cost was significantly higher in Group I compared with Group Ⅱ (32401.9 ± 1981.6 RMB *vs* 26961.9 ± 2293.8 RMB, P < 0.001).

CONCLUSION: Anvil insertion was faster and easier using the HDST technique compared with OrVil™, and was more cost-effective. There was no significant difference in safety.

Key words: Laparoscopy; Gastrectomy; Gastric cancer; Esophagojejunostomy; Cohort analysis

Core tip: Reconstruction of the digestive tract after total gastrectomy is technically difficult using laparoscopy. This study investigated two different methods to simplify this technique: a transorally inserted anvil (OrVil™) and the hemi-double stapling technique (HDST). The patients were randomized for comparison of these methods after laparoscopy-assisted radical total gastrectomy with D2 lymph node dissection. Both methods had similar safety and operation success. However, anvil insertion was faster and easier with HDST than with OrVil™, and was more cost-effective.

Wang H, Hao Q, Wang M, Feng M, Wang F, Kang X, Guan WX. Esophagojejunostomy after laparoscopic total gastrectomy by OrVil™ or hemi-double stapling technique. *World J Gastroenterol* 2015; 21(29): 8943-8951 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i29/8943.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i29.8943

INTRODUCTION

Surgery is the main treatment for gastric cancer[1]. Laparoscopy-assisted radical gastrectomy for gastric cancer has been used for more than 20 years[2]. The improvement of these techniques and apparatus have led to a gradual expansion of the available laparoscopy-assisted surgical methods for gastric cancer, allowing a more complete dissection of lymph nodes[3,4]. However, there are still some technical issues, and reconstruction of the digestive tract after total gastrectomy is one of these.

Delayed development of laparoscopic total gastrectomy is mainly attributed to the high technical requirements for laparoscopic esophagojejunostomy. Currently, many methods are available for reconstruction of the digestive tract after total gastrectomy[5-7]. An upper vertical midline incision with a length of about 5-7 cm in the abdominal wall is usually used to perform esophagojejunostomy after laparoscopic total gastrectomy. The length of the incision might even reach 8-11 cm in some obese patients, resulting in a more invasive treatment[8]. Side-to-side esophagojejunal anastomosis has been used for many years. This method has a large anastomotic diameter and anastomotic stricture is not easy to perform after surgery, but could solve some problems of esophagojejunostomy[7,9,10]. End-to-side esophagojejunal anastomosis is still a widely used anastomotic method[11]. However, some procedures are very difficult to perform, such as placement of the stapler anvil on the esophageal stump. If an open operation is performed, the first step of this process is to perform a purse-string suture of the lower edge of the esophagus. However, this procedure is difficult under laparoscopy, and could easily lead to potential problems in the operation, failure in anastomosis and prolonged surgery.

Many methods have been suggested for improving the placement of anvils under laparoscopy[12-15]; however, there is no consensus about their use. Currently, the major methods of anvil insertion involve transoral and intra-abdominal placements. The transorally-inserted anvil (OrVil™)[16] and the hemi-double stapling technique (HDST)[17] were recently developed. Although these two techniques have only been used for a short period, they have attracted much attention because they are simple and omit the need for a purse-string suture of the esophagus[18-21]. The OrVil™ technique inserts the stapler anvil through a transoral esophageal approach. A tube is connected with the central rod of the stapler. The tube is inserted in the esophagus and pulled out from the esophageal stump, and the anvil is placed under the guide of the tube. In the

HDST method, the anvil is inserted through the lower esophagus, and the needles and threads are pulled out from the anterior wall of the esophagus. Subsequently, the lower esophagus is closed, and finally, the anvil is pulled out from the anterior wall close to the esophageal stump guided by the needles and threads, thereby completing the anvil insertion. In this technique, the anvil is inserted in a lower-upper pattern, and purse-string suturing is not required. These two methods are skillfully designed, simple and practical, and they can be completely mastered after simple training. However, it is not clear which of these methods is the best one.

Therefore, this study used either OrVil™ or HDST anvil insertion methods to perform esophagojejunostomy after laparoscopic total gastrectomy and compared the effectiveness of these two methods in an open-label prospective cohort study. The results should provide important information on selection of the best method for anvil insertion for esopagojejunostomy in the clinical practice.

MATERIALS AND METHODS

Patients

Patients with gastric cancer and who were admitted to the Department of General Surgery, Drum Tower Hospital, Medical School of Nanjing University, Nanjing, China from May 2011 to October 2013 were approached for participation in this study. Inclusion criteria were: (1) willing to participate in the study; (2) male or female subjects aged $\leqslant$ 75 years; (3) newly diagnosed gastric adenocarcinoma confirmed by gastroscopy and histopathology; and (4) body mass index (BMI) $\leqslant$ 26.0 kg/m^2. Exclusion criteria were: (1) severe cardiac, hepatic or renal insufficiency, or hematopoietic dysfunction; (2) paraaortic lymph node metastasis or lymph node invasion to major blood vessels revealed by pre-operative or intra-operative exploration; (3) metastatic gastric cancer; (4) metastases to the liver, lung and other organ according to enhanced abdominal CT scan and chest X-ray; (5) tumor invasion into adjacent organs revealed by pre-operative examination or intra-operative exploration; (6) peritoneal dissemination; or (7) carcinoma of the gastric cardia involving the esophagus.

The study was approved by the Ethics Committee of the Drum Tower Hospital, Medical School of Nanjing University, Nanjing. Written informed consent was obtained from all participants.

Study design

This was an open-label prospective cohort study. After resection of the stomach and lymph node dissection, all subjects were randomized into two groups using a computer-generated random number table in a 1:1 ratio. Patients in Group Ⅰ underwent esophagojejunostomy using OrVil™, while patients in Group Ⅱ underwent HDST. Randomization was implemented using individual sealed envelopes (n = 84) prepared in advance by a statistician, and envelopes were opened by the surgeon according to the operation order. Researchers were blinded to the study grouping.

The primary endpoint was evaluation of the surgical outcome (operating time, time of digestive tract reconstruction and time of anvil insertion) and the medical cost of each operation (operation cost and total cost of hospitalization). The secondary endpoints were time to solid diet, post-surgical hospitalization time, time to defecation, time to ambulation and intra-operative blood loss. In addition, complications were assessed and compared.

Clinical data collection

Background demographic and clinical data were collected from the patients' medical records. The surgical evaluations (time for the procedures and blood loss) were collected during the procedure. The post-surgical information was collected by the clinical nursing staff. The patients were followed-up once every two months for the first postoperative year, and once every three months for the second postoperative year.

Surgical procedure

In accordance with other Asian countries, but in contrast to many Western countries, D2 lymphadenectomy was performed for all patients[1]. All surgeries were performed by the same surgeon and the same surgical team. Prior to the completion of this study, the surgeon and all members of the group had performed laparoscopic gastrectomy for more than 50 cases.

Patients were placed in the supine position, with legs wide apart. The surgeon stood on the left side of the patient, with the assistant on the right side and the laparoscope holder between the patient's two legs. A CO_2 pneumoperitoneum was established by CO_2 injection through an umbilical port, and the 10-mm port served as the observation port for the laparoscope. A 12-mm port was used on the left anterior axillary line below the costal margin, as the main operating port. A 5-mm port 5 cm to the left side of the umbilicus served as the auxiliary operating port, while a 12-mm port on the right anterior axillary line below the costal margin and a 5-mm port superior to the umbilicus on the right midclavicular line were used as the assistant operating ports.

Total gastrectomy and lymph node dissection were performed. The stomach was dissected along the left gastrocolic ligament, the roots of the left gastroepiploic vessels and short gastric arteries were ligated, and lymph nodes 4sa and 4sb were dissected. The right gastroepiploic artery and vein were ligated along the right pancreatic surface and the sixth group of lymph nodes was dissected. The stomach was dissected along the gastroduodenal artery and the common hepatic

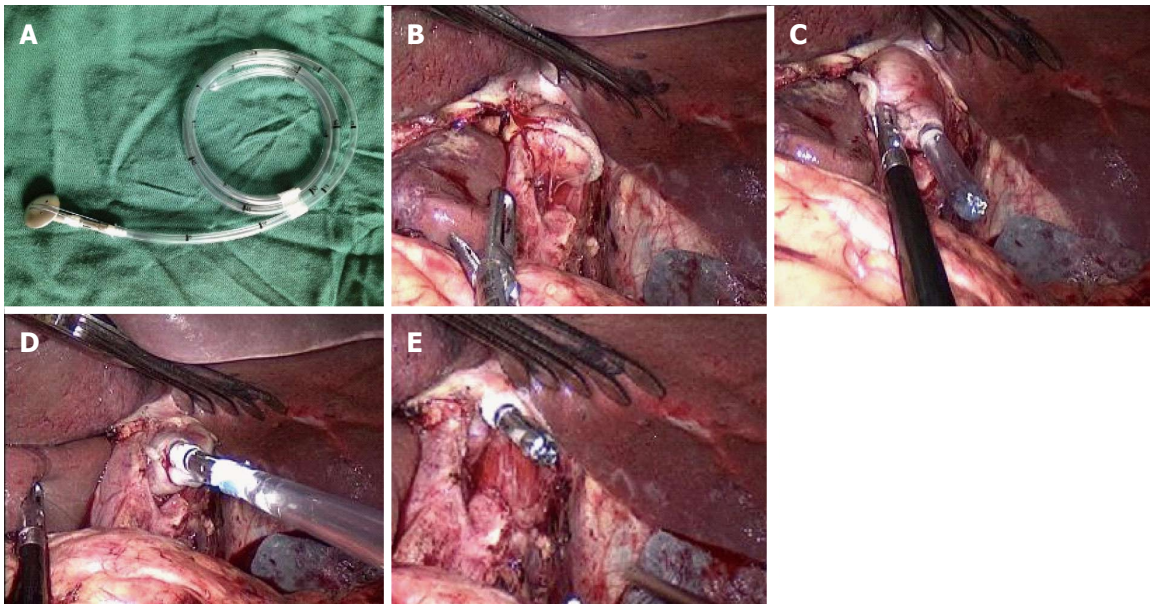

Figure 1 OrVil™ procedure. A: The central rod of the anvil connected with a tube; B: The lower esophagus was dissociated, and the esophagus was closed and cut; C: The tube of the OrVil™ system was transorally inserted into the esophagus, and the head of the tube was pulled out from the small hole at the end of the esophagus; D: The tube was pulled out until the anvil that connected with the end of the tube reached the esophageal stump; E: The connection line between the tube and anvil was cut down, and the tube was pulled out.

artery, and the root of the right gastric artery was ligated. Lymph nodes 5, 8a and 12a were dissected. The stomach was turned over to the head side, and the left gastric artery and vein along with the splenic artery were exposed and ligated. Lymph nodes 7, 9 and 11p were dissected. The duodenum was cut with a linear stapler (Ethicon Endosurgery; Cincinnati, OH, United States).

Anvil insertion into the end of the esophagus was performed using the OrVil™ system or HDST. Briefly, the OrVil™ technique was performed as follows. The lower edge of the esophagus was fully dissociated, and the esophagus 3 cm superior to the cardia was cut down using a linear stapler (Figure 1A-E). The tube of the OrVil™ system (OrVil™; Covidien, Mansfield, MA, United States) was transorally inserted into the esophagus with the anesthetist's assistance. Once the head of the tube reached the esophageal stump, a small port was pushed into the esophageal stump using an ultrasound scalpel. The tube was pulled out from the port until the anvil connecting with the end of the tube reached the esophageal stump. The connection line between the tube and the anvil was cut, and the tube was pulled out.

The HDST method was performed as follows (Figure 2A-E). The tip of the rod on the anvil was sutured with a needle containing sutures 4-5 cm in length. The prepared anvil was inserted into the abdomen. An incision of 2 cm in diameter was cut on the anterior wall of the cardia. The anvil was longitudinally inserted into the esophagus until the rod of the anvil was totally inserted into the esophagus and exceeded the tangent level of the esophagus. Then, the needle and thread were pulled out from the anterior wall of the esophagus. The esophagus was cut down using a linear stapler, and the needle and thread were further pulled outside until the rod of the anvil was completely pulled out from the anterior wall of the esophagus.

A Roux-en-Y esophagojejunostomy was performed. A 3.5-cm incision was made 2 cm to the left side of the umbilicus, and specimens were sampled *via* the incision on the abdominal wall. End-to-side jejunojejunal anastomosis was performed *via* the incision, and a jejunal portion of about 50 cm in length was retained. The circular stapler was inserted into the jejunum and temporarily fixed with rubber bands. The anastomotic device and small intestine were placed into the abdomen, and a pneumoperitoneum was re-established by clipping the abdominal wall with towel forceps. End-to-side esophagojejunal anastomosis was performed under a laparoscope, and the jejunal stump was closed using a linear stapler. Digestive tract reconstruction was performed, and a drainage tube was routinely placed beside the anastomotic stoma.

Statistical analysis

All data are expressed as mean ± standard deviation (SD). All statistical analyses were performed using SPSS 17.0 (IBM, Armonk, NY, United States). Continuous variables were compared using the Student's *t* test if the variances in both groups were equal; otherwise, the Welch's *t* test was used (time of post-surgical hospitalization). Categorical data (sex, TNM stage, tumor site) were compared with the Fisher's exact test. A P value < 0.05 was considered statistically significant.

The statistical methods of this study were reviewed by Dr Guan from Drum Tower Hospital, Medical School of Nanjing University, Nanjing.

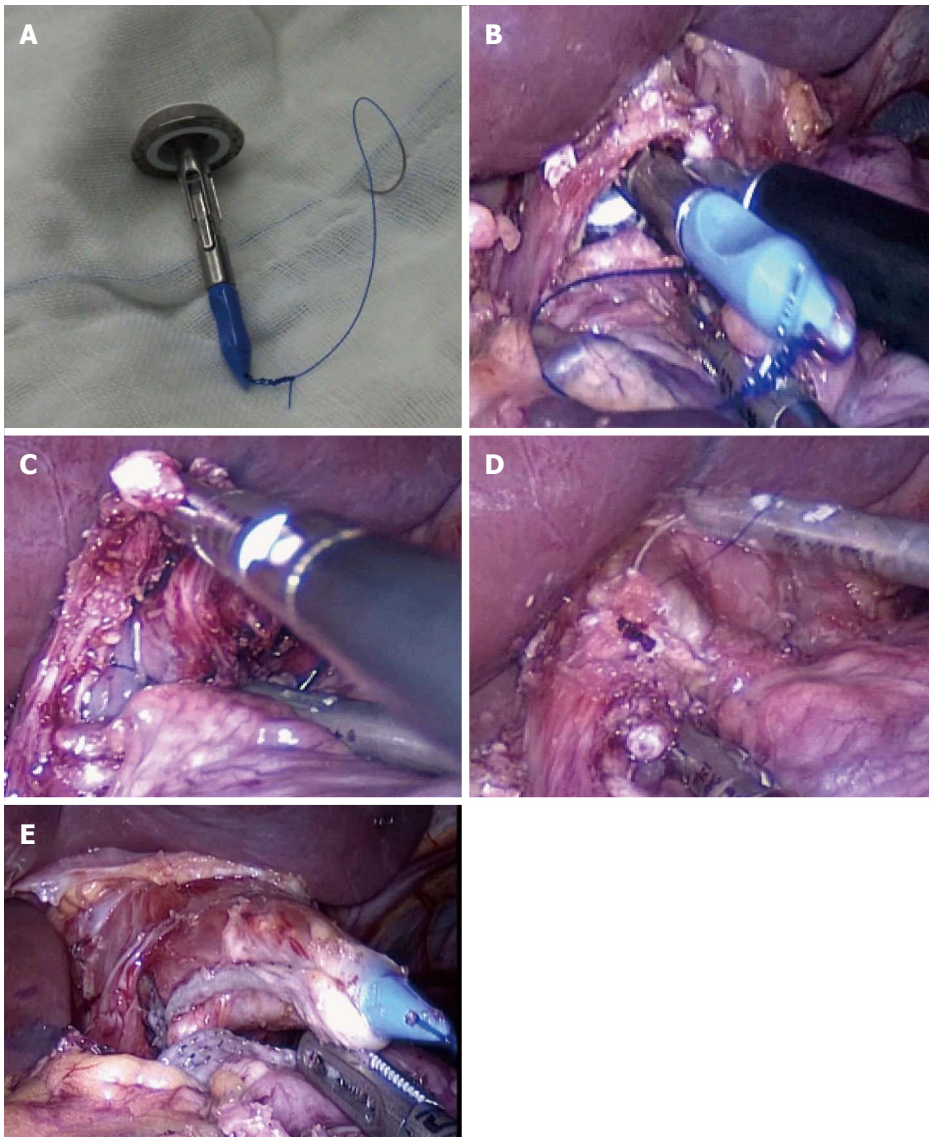

Figure 2 Hemi-double stapling technique procedure. A: The tail of the central rod of the anvil was sutured with sutures that contained a needle; B: The anvil with needle-containing sutures was inserted into the esophagus *via* the incision on the lower edge of the esophagus; C: The needle was inserted through the anterior wall of the esophagus, and the needle and sutures were pulled out from the anterior wall of the esophagus; D: The lower edge of the esophagus was closed off and cut down using a linear cutter; E: The sutures were further retracted until the central rod of the anvil was completely pulled out.

RESULTS

Patient enrollment

Figure 3 presents the patients' flowchart. Eighty-seven patients were initially included in the study, but three were excluded because of tumor invasion or metastasis. Therefore, 84 patients were finally included and randomized.

Baseline characteristics

There was no significant difference in age, BMI, tumor location and TNM stage between the two groups (Table 1). There were 24.4 and 26.7 lymph nodes dissected in groups Ⅰ and Ⅱ, respectively ($P > 0.05$).

Primary endpoint

The primary endpoint of the study was the surgical difficulty. All 84 patients underwent successful esophagojejunostomy, without conversion to laparotomy. The mean operative time was 287.8 ± 38.4 min in Group Ⅰ, including 147.7 ± 31.6 min for total gastrectomy and lymph node dissection, and 55.4 ± 15.7 min for digestive tract reconstruction. The mean operative time was 271.8 ± 46.1 min in Group Ⅱ, including 159.8 ± 33.8 min for total gastrectomy and lymph node dissection, and 47.8 ± 12.1 min for digestive tract reconstruction.

There was no significant difference in the mean operative time, and in the mean time for total gastrectomy and lymph node dissection between the two groups ($P > 0.05$). The mean time for stapler anvil insertion using the OrVil™ system was 18.7 ± 7.5 min, while it was 12.6 ± 4.7 min with HDST ($P < 0.05$), indicating that anvil insertion took less time when the

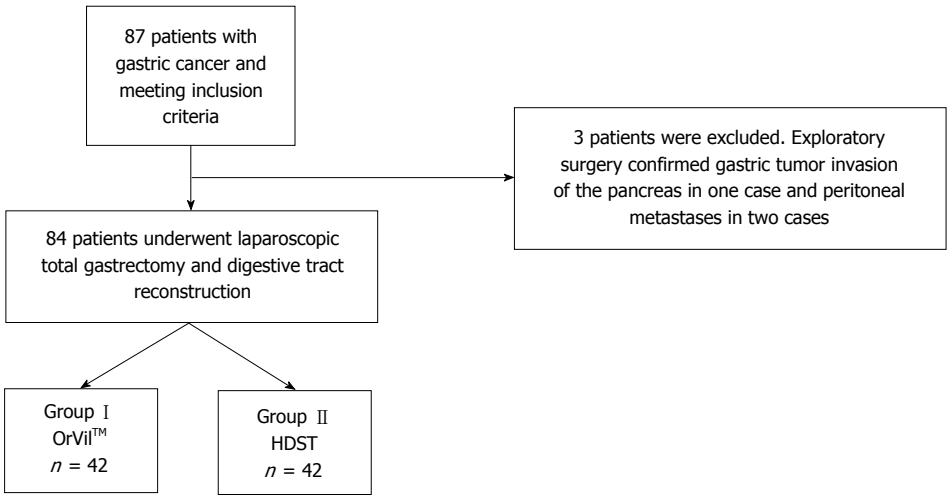

Figure 3 Patients' flowchart. Flowchart of the subject inclusion and allocation into group Ⅰ and group Ⅱ for the different insertion techniques for esophagojejunostomy. HDST: Hemi-double stapling technique.

Table 1 Clinicopathological characteristics

	OrVil™	HDST	*P* value
Sex (M/F)	31/11	27/15	0.345
Age	58.4 ± 8.0	56.5 ± 7.9	0.28
BMI (kg/m^2)	23.1 ± 2.5	22.5 ± 2.7	0.28
Resection margin (cm)	2.9 ± 0.7	2.8 ± 0.5	0.40
Length of surgical incision	3.9 ± 0.6	3.7 ± 0.7	0.38
Number of retrieved lymph nodes	24.4 ± 6.8	26.7 ± 5.5	0.09
TNM Stage			0.802
ⅠA	4	7	
ⅠB	13	12	
ⅡA	10	8	
ⅡB	7	10	
ⅢA	5	3	
ⅢB	3	2	
Tumor Site			0.165
Cardia and gastric fundus	31	25	
Body of stomach	11	17	

HDST: Hemi-double stapling technique; BMI: Body mass index; TNM: Tumor node metastasis.

Table 2 Comparison of surgery-related variables between the two groups

Variable	Group Ⅰ (*n* = 42)	Group Ⅱ (*n* = 42)	*P* value
Time of surgery (min)	287.8 ± 38.4	271.8 ± 46.1	0.09
Time of lymph node dissection and total gastrectomy (min)	147.7 ± 31.6	159.8 ± 33.8	0.09
Time of digestive tract reconstruction (min)	55.4 ± 15.7	47.8 ± 12.1	0.011[a]
Time of anvil insertion (min)	18.7 ± 7.5	12.6 ± 4.7	0.001[a]
Intra-operative bleeding volume (mL)	96.4 ± 32.7	88.2 ± 36.9	0.28
Time of defecation (d)	3.5 ± 0.9	3.2 ± 1.1	0.12
Time to get out of bed (d)	3.9 ± 0.7	3.6 ± 1.1	0.12
Time to post-surgical eating (d)	7.6 ± 1.4	8.0 ± 2.7	0.31
Time of post-surgical hospitalization (d)	10.6 ± 2.6	10.8 ± 3.5	0.80

[a]$P < 0.05$, Group Ⅰ *vs* Group Ⅱ. Data presented as mean ± SD.

HDST technique was used, and the mean time of the digestive tract reconstruction was, accordingly, shorter (Table 2).

The total costs of hospitalization were 73848.7 ± 11781.0 RMB in Group Ⅰ, including operation cost of 32401.9 ± 1981.6 RMB, and 70870.3 ± 14003.5 RMB in Group Ⅱ, including operation cost of 26961.9 ± 2293.8 RMB. The operation cost in Group Ⅱ was significantly lower than in Group Ⅰ ($P < 0.001$) but the total cost of hospitalization was not different between the two groups ($P = 0.296$) (Figure 4).

Secondary endpoints

In Group Ⅰ, mean intra-operative blood loss was 96.4 ± 32.7 mL, the mean time to defecation was 3.5 ± 0.9 d, the mean time to ambulation was 3.9 ± 0.7 d, the mean time to post-surgical eating was 7.6 ± 1.4 d and the mean time of hospitalization was 10.4 ± 2.6 d. In Group Ⅱ, the mean intra-operative bleeding volume was 88.2 ± 36.9 mL, the mean time to defecation was 3.2 ± 1.1 d, the mean time to ambulation was 3.6 ± 1.1 d, the mean time to post-surgical eating was 8.0 ± 2.7 d and the mean time of hospitalization was 10.8 ± 3.5 d. There was no significant difference in all these parameters between the two groups (all $P > 0.05$) (Table 2). The mean distance from the surgical margin was 2.9 ± 0.7 cm and 2.8 ± 0.5 cm on proximal esophagus to the cancer in the patients with carcinoma of gastric cardia from Groups Ⅰ and Ⅱ, respectively.

Adverse events

No residual cancer tissue was found in all cases. A high post-surgical short-term therapeutic efficacy was achieved in both groups with no bile reflux.

Intra-operative complications occurred in some

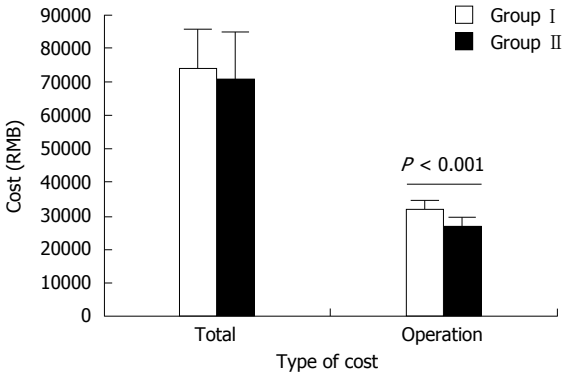

Figure 4 Comparison of the total cost of hospitalization and operation between the two groups. Dark grey represents the cost using the OrVil™ system (Group Ⅰ) and light grey represents the cost using the hemi-double stapling technique (HDST) method (Group Ⅱ). The cost of operation using the OrVil™ system was significantly greater than that of using the HDST method (P < 0.001, Group Ⅰ *vs* Group Ⅱ).

patients. After surgery, two cases with atelectasis, two cases with incision dehiscence and three cases with throat pain were observed in Group Ⅰ. One case had pleural effusion and one case had esophageal-jejunal anastomotic fistula (diagnosed by radiographic examination) in Group Ⅱ, which was cured with drainage and enteral nutrition for 18 d.

All patients were followed up for 10-28 mo after surgery, with a median follow-up period of 16 mo. During follow-up, other adverse events occurred. Four patients, two in each group, suffered from an esophageal-jejunal anastomotic stenosis (difficulty in swallowing, which was confirmed by esophagography) that was relieved by endoscopic dilatation. In Group Ⅰ, one case had peritoneal implantation metastases 7 mo postoperatively and died 14 mo postoperatively. Another case had a liver metastasis and entered remission with chemotherapy. In Group Ⅱ, one case had peritoneal implantation metastases 9 mo postoperatively and entered remission with chemotherapy.

DISCUSSION

The aim of the present trial was to compare two different methods of anvil insertion for esophagojejunostomy after laparoscopic total gastrectomy, primarily in terms of surgical difficulty. All procedures were successfully completed during the 84 total gastrectomy surgeries in patients with gastric cancer. In addition, no technical problems occurred in any case during the reconstruction of the digestive tract and none of the cases needed conversion to laparotomy or expansion of the surgical incision, indicating that these two methods had a high reliability and stability.

Novel methods have been introduced for esophagojejunostomy because of the technical difficulties of this procedure in a laparoscopic setting. Purse-string suturing is a difficult technique to perform in a narrow space with a restricted view, which requires experience and skill from the surgeon. When purse-string suturing is required, a lot of surgical time is consumed. Therefore, most surgeons are reluctant to perform such procedures. Both the OrVil™ system[22-24] and HDST[25,26] have been shown to simplify esophagojejunostomy, with high success rates and few requirements for transfer to laparotomy. To our knowledge this is the first randomized study to compare these two methods after total gastrectomy, although one previous study compared these methods on a smaller cohort alongside a conventional anvil head method and side-to-side esophagojejunostomy with a linear stapler[21]. That study showed that surgery with OrVil™ was similar in time course to a more traditional method and concluded that none of the methods tested were entirely satisfactory. In the present study, the mean time of digestive tract anastomosis was 55.4 min using the OrVil™ system and 47.8 min using the HDST technique. In addition, the mean time for stapler anvil insertion was 18.7 min using the OrVil™ technique and 12.6 min using the HDST technique. Both of these techniques completed anvil insertion in a short time period, indicating that they were simple and easy to perform. Anvil insertion using the OrVil™ technique took a longer time than the HDST technique, which might be attributed to transoral placement procedures. In addition, the flexible tube operated by the anesthesiologist was difficult to control. In order to enable the head of the tube to be fixed in a good position, repeated adjustment was required that led to a longer operation time. However, due to omission of purse-string suturing, these two methods exhibited a significant superiority over the traditional purse-string suture methods in terms of the time of operation, which in our experience take approximately 20-25 min longer.

An important factor in survival after total gastrectomy is achieving a negative surgical margin. This is influenced by a number of factors, but most importantly the extent of the tumor and the extent of surgery[27]. The mean distance between the surgical margin on the proximal esophagus and the tumor is an important marker for a negative surgical margin[28]. If an open operation is performed, the surgical margin of the esophagus can reach 3-5 cm superior to the cardia. In the present study, among the 56 cases with carcinoma involving the gastric cardia, the mean distance between the surgical margin on the proximal esophagus and the cancer was about 3 cm, and no residual cancer tissues were observed, which ensured the completion of tumor resection.

Comparison of open and laparoscopic techniques for total gastrectomy suggests that these methods have similar outcome and success rates, but laparoscopic total gastrectomy can be associated with an increased complication rates in comparison with open surgery[29]. The most common of these complications is an anastomotic fistula, although recent laparoscopic methods have shown decreased rates as the techniques have

improved[30]. Of the 84 patients who underwent esophagojejunostomy, anastomotic fistula occurred in only one case from Group Ⅱ. This case was diagnosed as esophageal-jejunal anastomotic micro-fistula using radiographic examination and was cured with post-surgical drainage and enteral nutrition. If anastomosis is unsatisfactory, it is suggested that the suture be strengthened and the drainage tube be routinely indwelled beside the anastomotic stoma. Radiographic examinations should be performed in some suspected patients prior to removal of the drainage tube in order to exclude anastomotic fistula.

Anastomotic stenosis is another complication commonly observed in patients after undergoing laparoscopic total gastrectomy. In the present study, 4 out of 84 patients developed anastomotic stenosis. Shim *et al*[21] reported that anastomic stenosis occurred in 5 out of 26 patients after esophagojejunostomy using the OrVil™ and HDST techniques. Umemura *et al*[31] reported that the incidence of stenosis after esophagojejunostomy using a linear stapler was only 1.8%. Therefore, we suggest that the incidence of anastomotic stenosis after esophagojejunostomy using circular stapler methods was higher than that using a linear stapler. However, further study is necessary to address this issue.

Based on previous reports, some surgeons have made several modifications to these two surgical methods with the aim of overcoming the corresponding shortcomings. Indeed, the OrVil™ technique often results in a dog ear, and Hirahara *et al*[26] tried to solve this issue with a loop-shaped thread wrapped around the esophageal stump opening. With this modification, esophagojejunostomy was completed without a dog-ear. This approach may decrease the incidence of anastomotic fistula, but studies with larger sample size are required to support this assertion due to the small number of cases in this study. Muguruma *et al*[19] described a similar technique based on the procedure described by Omori *et al*[17], except that they used the OrVil™ anvil instead of the ECS25 (Ethicon Endo-Surgery stapler). It was found that the OrVil™ anvil was much easier to use and that surgeons with relatively little experience in gastric laparoscopy were able to conduct the operation and avoid complications. The median operative time was 318 min including 5 min for the placement of the anvil on the esophageal stump. Compared with these two retrospective studies, the present study focused on the prospective comparison between the two methods. We believe that these two methods for esophagojejunostomy will gain an increasing recognition, and will be widely used by more and more surgeons.

Compared with the HDST technique, the OrVil™ system requires a specific stapler and has a higher cost, while the HDST technique does not need a specific stapler and has a relatively low cost. Therefore, the use of the HDST technique might be more practical in undeveloped areas of the world.

The present study has some limitations. As the main purpose of the study was to evaluate two different methods of anvil insertion for esophagojejunostomy, we did not collect detailed data on tumor differentiation status, since these would not generally impact upon the surgical difficulty. If these data had been collected, a more detailed comparison between the groups and long-term outcomes could be performed. A larger multicenter study would also add more data and provide more convincing evidence for the most suitable anvil insertion method. A longer follow-up period would provide more information on the long-term effects of surgery and should be considered in future studies.

In summary, we conclude that the OrVil™ and HDST techniques were simple and reliable without any significant differences in safety and difficulty of operation. Both were reliable techniques for laparoscopic esophagojejunostomy.

COMMENTS

Background

Laparoscopic total gastrectomy is still not widespread because of the technical difficulty of the reconstruction. Although various types of esophagojejunal anastomosis are described in the literature, the optimal procedure has yet to be established. The placement of the stapler anvil is one of the limiting steps of laparoscopic total gastrectomy.

Research frontiers

There are many suggested methods for improving the placement of anvils under a laparoscope. The transorally-inserted anvil (OrVil™) and the hemi-double stapling technique (HDST) are recently developed methods of anvil insertion. However, there is no consensus on the best technique.

Innovations and breakthroughs

Previous reports have been issued on the feasibility of OrVil™ and HDST. To the best of our knowledge, this is the first report that compares the two types of anastomosis after laparoscopic total gastrectomy.

Applications

Both methods had similar safety and operation success. However, anvil insertion was faster and easier with HDST than with OrVil™, and was more cost-effective.

Peer-review

This study is excellent with adequate sample size records. Case sheets should be used to check the genuineness of findings as this study could change treatment as well as the technique used. This article can be accepted with these conditions and with the corrections mentioned to the author.

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SYSTEMATIC REVIEWS

Effects of cereal fiber on bowel function: A systematic review of intervention trials

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Data sharing statement: No additional data are publically available. Interested readers can contact the first author.

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Abstract

AIM: To comprehensively review and quantitatively summarize results from intervention studies that examined the effects of intact cereal dietary fiber on parameters of bowel function.

METHODS: A systematic literature search was conducted using PubMed and EMBASE. Supplementary literature searches included screening reference lists from relevant studies and reviews. Eligible outcomes were stool wet and dry weight, percentage water in stools, stool frequency and consistency, and total transit time. Weighted regression analyses generated mean change (± SD) in these measures per g/d of dietary fiber.

RESULTS: Sixty-five intervention studies among generally healthy populations were identified. A quantitative examination of the effects of non-wheat sources of intact cereal dietary fibers was not possible due to an insufficient number of studies. Weighted regression analyses demonstrated that each extra g/d of wheat fiber increased total stool weight by 3.7 ± 0.09 g/d (P < 0.0001; 95%CI: 3.50-3.84), dry stool weight by 0.75 ± 0.03 g/d (P < 0.0001; 95%CI: 0.69-0.82), and stool frequency by 0.004 ± 0.002 times/d (P = 0.0346; 95%CI: 0.0003-0.0078). Transit

time decreased by 0.78 ± 0.13 h per additional g/d (P < 0.0001; 95%CI: 0.53-1.04) of wheat fiber among those with an initial transit time greater than 48 h.

CONCLUSION: Wheat dietary fiber, and predominately wheat bran dietary fiber, improves measures of bowel function.

Key words: Comprehensive review; Dietary fiber; Wheat bran; Cereal; Bowel function

Core tip: This comprehensive review evaluates available data on the effects of intact cereal dietary fiber on bowel function and provides a quantitative summary of the effect of intact wheat fiber on bowel function using weighted regression analysis. Insufficient observations were available from non-wheat cereals for quantitative analysis. Results found an increase in total stool weight of 3.7 ± 0.09 g per gram intact wheat fiber. Transit time decreased by approximately 45 min per gram intact wheat fiber when initial transit time was greater than 48 h. Therefore, intact wheat dietary fiber, predominantly from wheat bran, improves bowel function.

de Vries J, Miller PE, Verbeke K. Effects of cereal fiber on bowel function: A systematic review of intervention trials. *World J Gastroenterol* 2015; 21(29): 8952-8963 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i29/8952.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i29.8952

INTRODUCTION

Composition, consistency, frequency, and weight of bowel movements are key indicators of intestinal and digestive health[1]. Abnormalities in these factors serve as diagnostic criteria for prevalent gastrointestinal disorders such as functional constipation[2,3]. According to the most widely accepted criteria (Rome Ⅲ)[2], characteristics of functional constipation include defecation associated with straining, hard stools, a sensation of incomplete evacuation or anorectal obstruction, manual maneuvering to facilitate defecation, and less than three stools per week. Normal, healthy bowel function, on the other hand, is characterized by soft, regularly shaped stool that is easy to pass, and bowel movements occurring twice per day to three times per week, depending on the individual[4]. Functional constipation is a heterogeneous and common disorder that affects apparently healthy populations[5]. Reports of prevalence vary widely, depending on definition, demographic factors, and sampling[6-9], but could be as high as 27%[3].

Constipation and digestive discomfort have multiple etiologies[3], including certain medications, abuse of laxatives, hormonal disorders and inadequate dietary fiber intakes. Suboptimal dietary fiber consumption is increasingly a global concern, as average intakes are well below recommendations across many countries[10,11]. This creates considerable clinical and public health opportunities to identify strategies that will increase dietary fiber intakes to improve bowel function and help prevent digestive disorders. In addition, increasing dietary fiber consumption offers a safer and cost-effective alternative to laxatives for preventing or alleviating symptoms of constipation[5].

Dietary fiber is naturally present in different food groups, including cereals, vegetables, fruits, beans, and peas. This review provides an overview of intervention studies examining intact cereal dietary fibers (ICDF), which are derived from any part of the cereal plant, including the kernel, hull, or stalk and are minimally processed, although some degree of processing may be required to obtain the fiber-rich portion of the kernel (*e.g.*, milling of bran) or to improve food functionality or safety (*e.g.*, pearling, grinding, or bleaching). In contrast, fibers that are extracted, isolated, or made by chemical or enzymatic means, such as the synthesis of fibers from endosperm starch or the enzymatic hydrolysis of long chain fibers into oligosaccharides are not ICDF and are not included in this analysis. Cereal bran, the hard outer layer of a grain kernel, is a highly concentrated source of dietary fiber: per 100 g, wheat bran contains 43 g fiber, rice bran contains 21 g fiber, and oat bran contains 15 g fiber[12].

Although a large body of literature supports a role of ICDF, predominately wheat bran fiber[5,13], in promoting normal, healthy bowel function through increasing stool weight, past reviews were conducted more than two decades ago[14,15]. Since that time, a number of intervention studies have been published. In addition, less is known about the effects of wheat fiber on other measures of bowel function or the effectiveness of other ICDF such as those from oat, barley, rice, corn, and sorghum. Therefore, the purpose of the present study was to review, evaluate, and quantitatively summarize results from published intervention studies that examined the effects of ICDF on parameters for healthy bowel function, including stool wet weight, stool dry weight, percentage water in stool, stool frequency, intestinal transit time, and stool consistency. Although the heterogeneity of included studies does not allow for a meta-analytical approach, a quantitative estimate using weighted regression analysis on indicated parameters is provided on the pooled results of available studies.

MATERIALS AND METHODS

Literature search and study selection

A comprehensive literature search using PubMed

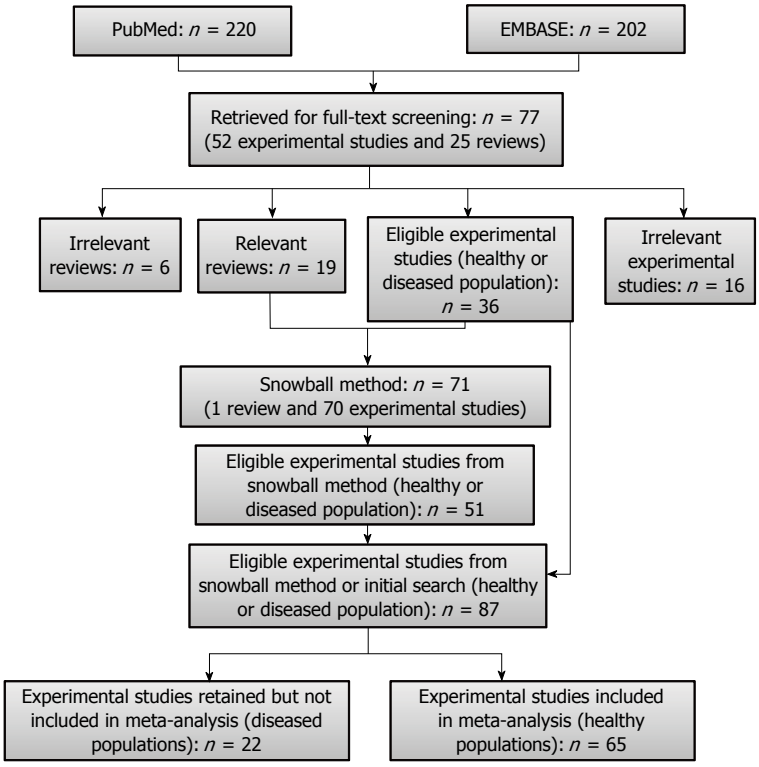

Figure 1 Literature search flow diagram.

and EMBASE was performed to identify intervention studies in human populations through 6 October 2012 (PubMed) and 18 October 2012 (EMBASE) with no lower date limit. The full search string used in each database is available in the Online Data Supplement (Appendix 1). A combination of free text terms, with different spellings and designed to capture relevant cereals and grains, fiber or bran, and relevant bowel function outcomes (*e.g*., stool, transit, volume, and bulk), was used. Supplementary literature searches involved examining the reference lists of all relevant studies and pertinent reviews to identify articles not captured in the initial search. The search flow is illustrated in Figure 1. The review was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines[16]. The PRISMA checklist is available in the Online Data Supplement (Appendix 2).

Interventions were considered eligible if the following criteria were met: (1) the study was performed with an ICDF; (2) the study was conducted in a human population aged > 1 year; (3) a relevant outcome measurement of bowel function, including total stool weight, stool dry weight, percentage stool water, number of bowel movements per day, consistency of stools, or transit time, was examined; and (4) the publication was written in the English language. Study populations with underlying gastrointestinal disorders, such as constipation, diarrhea, irritable bowel syndrome, diverticular disease, or ulcerative colitis, were eligible for inclusion in the search strategy and data extraction, but were not included in the present quantitative analyses. Both controlled and uncontrolled trials were included in this systematic review. Two independent reviewers (de Vries J and Verbeke K) screened the titles and abstracts for relevance to the systematic review to ensure quality-control. Potentially eligible articles were reviewed jointly to resolve any discrepancies.

Data extraction and quality assessment

The following general study information was extracted using FileMaker Pro software: first author; hypothesis; sex; sample size; study design; duration; physiological characteristics of participants; details of the intervention; details of the control group; background diet (including fiber content); fiber intervention; total fiber intake (background diet fiber plus fiber intervention); dose of food or ingredient in intervention; measured outcome parameter; method used to measure outcome parameter; and description of any adverse events. Outcome data for total stool weight (g/d), stool dry weight (g/d), percentage water in stools, number of bowel movements per day, consistency of stools, and transit time (h) were extracted, and included baseline and trial end values, change-from-baseline values, statistical significance of change values, and differences in the trial end value between the intervention and control arm. Lowest effective dose was identified by visual inspection of the data as reported in the individual studies.

Study quality was assessed through assignment of

scores according to two appraisal systems: (1) criteria developed by the FSANZ for the review of publications that are considered to support submitted health claims (0-15 points)[17]; and (2) criteria for human intervention studies as described by Welch *et al*[18] (0-20 points).

Statistical analysis

The included publications report on intervention studies with a diversity of study designs. Therefore, a meta-analytical approach according to PRISMA criteria was not feasible. Instead, the potential effect of ICDF on bowel function parameters was quantitatively estimated by a weighted regression on the results of those ICDF that had more than 5 observations per parameter. A weighted regression by sample size was chosen because not all publications reported SD on their results.

Means, standard deviations, and 95%CI for ICDF dose (g/d) and bowel function parameters were generated. Weighted regression analyses, in which data from each published study were weighted by the number of subjects used in the study, was performed using SAS version 9.2 (Cary, North Carolina, United States). The regression analysis was not forced through zero because the intercept was different from zero (3.06 ± 1.52 g; $P < 0.0439$; 95%CI: 0.08-6.03). Stool consistency was an eligible outcome, but due to the diversity of both the methods used to estimate stool consistency and the qualitative reporting of results, weighted regression analysis was not possible. For the analysis of total transit time, a multivariable weighted regression analysis was performed to account for differences in the relationship with the intervention fiber amount that depended upon the initial transit time. Comparisons of the effects of wheat fiber *vs* other ICDFs on bowel function parameters were not feasible due to a limited number of studies examining other ICDFs.

Data used for the control group differed according to the type of study. For placebo-controlled trials, data from the control arm was used. The control in these studies was most often white wheat, a usual diet, or a gelatin capsule. In some cases, a positive control, such as a laxative, cellulose, wheat bran (if another type of ICDF was examined), or another cereal was used. For uncontrolled trials, the baseline values of the intervention group were used as the control. Some studies conducted a dose-response intervention, in which case the lowest dose was considered the control.

RESULTS

Study characteristics

A flow diagram of the literature search is shown in Figure 1. The literature search included both healthy and diseased populations until the final stage, at which time the studies conducted in healthy populations, were separated from studies conducted in diseased populations. The search yielded 220 references in PubMed and 202 references in EMBASE, of which 77 articles were retained for full-text screening and reference list review. The 77 articles included both original experimental research publications (n = 52) and reviews (n = 25). Thirty-six of the experimental studies were deemed eligible and 19 of the review articles were deemed relevant for screening of reference lists (snowball method). Overall, screening of reference lists from all relevant review articles and eligible experimental studies resulted in 71 additional articles (1 review and 70 experimental studies) that subsequently underwent full-text screening. Fifty-one of the 71 articles were eligible for inclusion. Therefore, the 51 eligible experimental studies identified by the snowball method and the 36 eligible experimental studies identified in the initial search resulted in a combined total of 87 experimental studies, 65 of which were conducted in generally healthy populations and therefore included in the quantitative analyses. From the 65 studies, 87 study arms examined the effect of ICDF on total fecal weight, 47 on dry fecal weight, 36 on percentage fecal water, 43 on stool frequency/bowel movements, and 57 on transit time.

Primary characteristics, including the first author, publication year, sex distribution of study population, type of study design, and the specific ICDF evaluated, of the 65 interventions are provided in the Online Data Supplement (Appendix 3)[11,19-82]. Fifty-seven percent of the studies were placebo-controlled, 32% were randomized, and 6% were single- or double-blinded. Wheat fiber, and primarily wheat bran fiber (90% of wheat fiber studies), was the most common dietary fiber provided in the intervention with 75 observations in 65 intervention studies. Only 13 of the observations were ICDF from other sources, including corn (n = 4), barley (n = 3), rye (n = 2), oat (n = 1), rice (n = 1), and sorghum (n = 1). Most publications, also the more recent ones, provide insufficient details for an adequate description of the dietary fiber sources used.

Stool bulking, stool frequency, and transit time

Table 1 shows the number of comparisons for different ICDFs and different bowel function outcomes. It also presents the level of fiber provided across the interventions. Table 2 presents the mean ± SD and 95%CI effects, plus ranges from the individual studies, of the fiber intervention on total stool weight (g/d), dry stool weight (g/d), percentage water in stool (%), and stool frequency (times/d), as well as the average fiber intakes provided in the interventions for each of these outcomes for wheat, barley, and corn. Table 2 also shows results from the weighted regression analysis of wheat fiber (per g/d), compared to control, on change in total and dry stool weight, stool frequency (number of defecations/day), and transit time (h). The mean effects and weighted change on bowel

Table 1 Summary of comparisons for different intact cereal dietary fibers and bowel function outcomes

	Source of intact fiber						
	Wheat	Barley	Corn	Oat	Rice	Rye	Sorghum
Total stool weight							
Observations[1], *n*	75	3	4	1	2	2	1
Fiber intervention (g/d), mean ± SD or range[2]	15.2 ± 8.3	10.2, 23	6.0, 42	14.3	17.1, 20.7	13, 20.6	2.5
Dry stool weight							
Observations, *n*	40	1	3	1	1	1	-
Fiber intervention (g/d), mean ± SD or range[2]	14.7 ± 8.5	21	6, 42	14.3	20.7	20.6	-
Fecal water							
Observations, *n*	30	3	2	-	1	-	-
Level of fiber interv. (g/d), mean ± SD or range[2]	16.0 ± 7.4	10.2, 23	15, 42	-	20.7	-	-
Stool frequency							
Observations, *n*	34	2	2	-	2	2	1
Fiber intervention (g/d), mean ± SD or range[2]	13.6 ± 6.4	21, 23	15, 42	-	17.1, 20.7	20.6, 36.4	2.5
Transit time							
Observations, *n*	52	-	-	1	2	1	1
Fiber intervention (g/d), mean ± SD or range[2]	14.8 ± 8,6	-	-	2.7	17.1, 20.7	20.6	2.5

[1]May include > 1 observation from studies examining > 1 dose of intact cereal dietary fiber; [2]Fiber intakes are shown as mean ± SD of all observations if > 5 observations were available, the range of values from individual studies if 2-4 observations were available, and a single estimate if only one observation was available.

Table 2 Fiber intakes and effects on total stool weight, dry stool weight, percentage water in stool, stool frequency, and transit time

	Source of intact cereal dietary fiber		
	Wheat	Barley	Corn
Total stool weight			
Observations, *n*	75	3	4
Fiber (g/d), mean ± SD or range	15.2 ± 8.3	10.2-23	6.0-42
Total effect (g/d), mean ± SD or range	65.4 ± 37.8	49.6-65	1.2-96.3
Average fecal bulking index, Δ in g/d stool weight per g/d fiber	4.7 ± 2.7	3.6 ± 2.4	2.1 ± 1.5
Fecal bulking index by regression, Δ in g/d stool weight per g/d fiber	3.67 ± 0.09[b] (3.50-3.84)	-	-
Dry stool weight			
Observations, *n*	40	1	3
Fiber (g/d), mean ± SD or range	14.7 ± 8.5	-	6-42
Total effect (g/d), mean ± SD or range	14.4 ± 9.4	-	4.8-31
Fecal bulking index by regression, Δ in g/d stool weight per g/d fiber	0.75 ± 0.03[b] (0.69-0.82)	-	0.7-0.9
Fecal water			
Observations, *n*	30	3	2
Fiber (g/d), mean ± SD or range	16.0 ± 7.4	10.2-23	-
Total effect by regression (Δ% water), mean ± SD or range	1.5 ± 2.1	-1.8-10	-
Stool frequency			
Observations, *n*	34	2	2
Fiber (g/d), mean ± SD or range	13.6 ± 6.4	-	-
Total effect (times/d), mean ± SD or range	0.34 ± 0.23	-	-
Frequency index by regression, Δ in times/d per g/d fiber	0.004 ± 0.002[a] (0.003-0.078)	-	-
Transit time			
Observations, *n*	52	0	0
Fiber (g/d), mean ± SD	14.8 ± 8,4	-	-
Δ in h per g/d fiber by regression (those with initial transit time between 24-48 h)	0.78 ± 0.13[b] (0.53-1.04)	-	-
Δ in h per g/d fiber by regression (those with initial transit time between 48-96 h)	-0.75 ± 0.04[b] (-0.84- -0.67)	-	-

[a]$P < 0.05$, [b]$P < 0.01$ *vs* control.

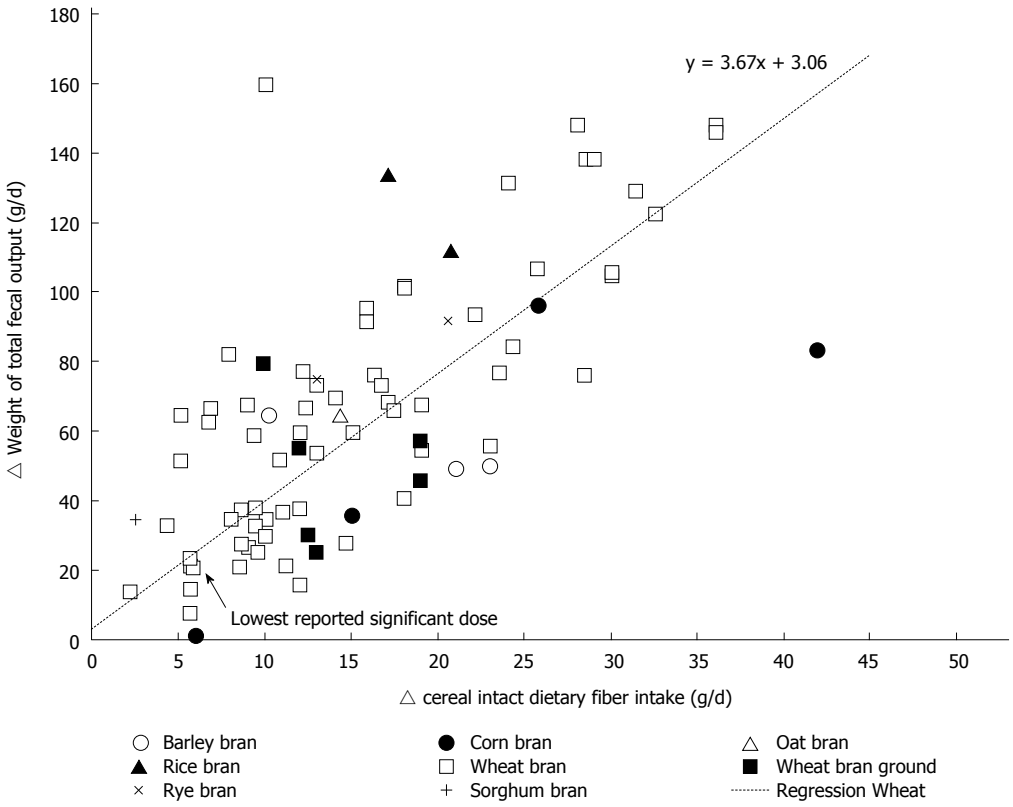

Figure 2 The delta weight of total fecal output (g/d) related to the amount of intact cereal dietary fiber intervention (g/d) in healthy individuals.

function parameters among interventions that used ICDF from barley and corn were not estimated given the limited number of observations (< 5 observations were available for each). The data of oat, rice, rye, and sorghum ICDF on these parameters from the individual studies are listed in the supplemental information (Appendix 4).

Among the studies included in the quantitative analysis (wheat fiber studies), mean fiber intakes ranged from 13.6 ± 6.4 g/d among studies that examined stool frequency to 16.0 ± 7.4 g/d among studies that investigated percentage water in stool. On average, the wheat fiber intervention increased total stool weight by 65.4 ± 37.8 g/d, dry stool weight by 14.4 ± 9.4 g/d, percentage water in stool by 1.5% ± 2.1%, and stool frequency by 0.34 ± 0.23 bowel movements per day.

The weighted changes per g/d of wheat fiber intake were as follows: an increase of 3.7 ± 0.09 g/d (P < 0.0001; 95%CI: 3.50-3.84) for total stool weight; an increase of 0.75 ± 0.03 g/d (P < 0.0001; 95%CI: 0.69-0.82) for dry stool weight. Weighted regression analysis of the results of all studies did not reveal an effect of the fiber intervention on transit time. Upon stratification by baseline transit time, an increase of 0.78 ± 0.13 h/g (P < 0.0001; 95%CI: 0.53-1.04) of wheat fiber was observed among those with an initial transit time of 24-48 h, and a decrease of 0.75 ± 0.04 h/g [P < 0.0001; 95%CI: (-0.84) - (-0.67)] of wheat fiber was observed among those with an initial transit time of 48-96 h. Individual study data on the change in the bowel function parameters per gram of wheat fiber intake are shown in Figure 2 for total stool weight, Figure 3 for dry stool weight, Figure 4 for percentage water in stool, and Figure 5 for stool frequency. The lowest effective dose of wheat fiber that significantly increased fecal output, as reported in one of the included individual intervention studies, was 5.7 g/d (P < 0.05)[53].

DISCUSSION

The present review provides the most comprehensive evaluation to date on the effects of ICDF on multiple measures of bowel function. Wheat fiber, and primarily wheat bran fiber, was found to improve measures of bowel function, including total stool weight, dry stool weight, and stool frequency, as well as intestinal transit time among those with an initial transit time greater than 48 h.

Wheat bran fiber is the most extensively studied cereal fiber for measures related to bowel function[5,13], with the first study dating back more than 90 years[82]. Leading nutrition and health authorities, including the US Institute of Medicine[83], Health Canada[84], and the European Food Standards Agency (EFSA)[85], have concluded that wheat bran fiber increases stool bulking and shortens intestinal transit time. In 2010, EFSA provided a Scientific Opinion[85], wherein an unequivocal cause and effect relationship between the consumption

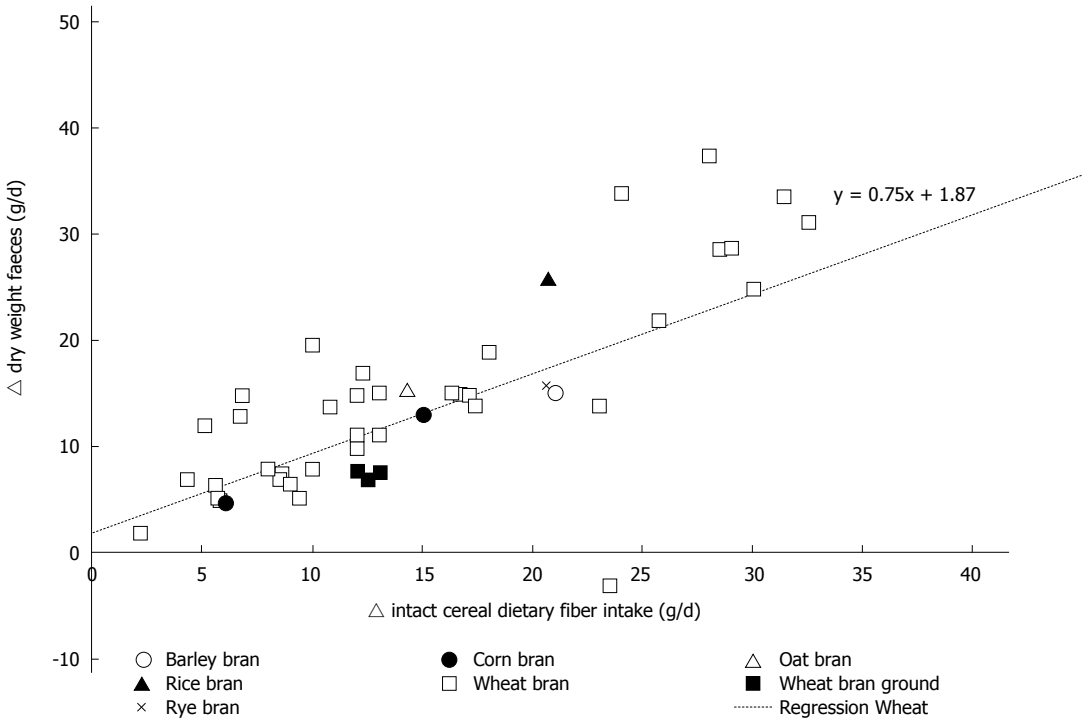

Figure 3 The delta weight of dry fecal output (g/d) related to the amount of intact cereal dietary fiber intervention (g/d) in healthy individuals.

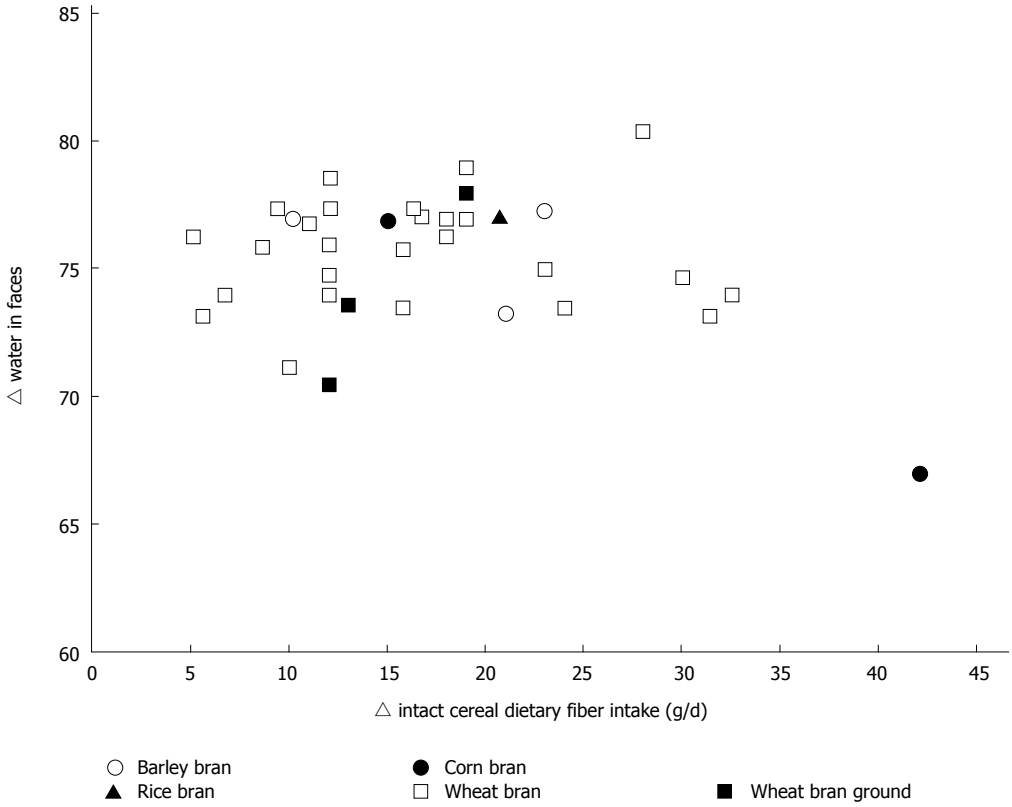

Figure 4 The % fecal water related to the amount of intact cereal dietary fiber intervention (g/d) in healthy individuals.

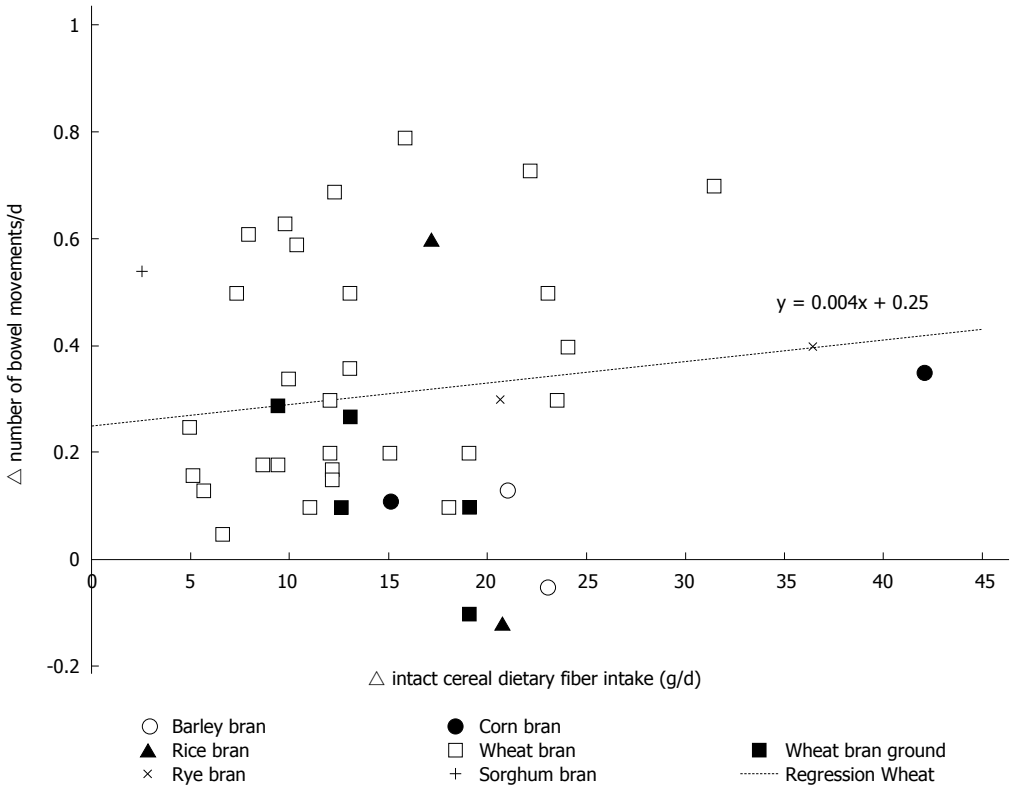

Figure 5 The delta number of bowel movements related to the amount of intact cereal dietary fiber intervention (g/d) in healthy individuals.

of wheat bran fiber and an increase in stool bulk and intestinal transit time was concluded and two health claims in relation to these intestinal functions were passed. A health claim was also approved by the Canadian Food Inspection Agency after the agency concluded that wheat bran promotes laxation and regularity[84]. Furthermore, wheat bran is considered the benchmark against which other fibers are compared for their effects on regularity[84]. Compared to wheat bran fiber, less is known concerning the effects of other sources of ICDF on bowel function, largely because far fewer studies have been conducted on other ICDF.

Given the heterogeneity in study designs utilized in the individual studies, a weighted regression, rather than a traditional meta-analysis, was considered to be a superior method to examine the effect of wheat fiber on stool parameters. The average fecal bulking index, reported in Table 2, provides an indicative estimate of effect of ICDF from wheat (75 observations), barley (3 observations) and corn (4 observations) on total fecal bulking. These results indicate that wheat bran might have the best properties to increase total fecal bulk.

Different sources of dietary fiber are not equal in their functionality and effects on bowel function, as evidenced by Health Canada using wheat bran as the gold standard fiber[84]. Cummings[14] evaluated nearly 100 interventions, published from 1932 to 1991, on dietary fiber and fecal weight, and compared the effectiveness of different sources of fiber. Among 41 interventions that examined wheat fiber-which consisted largely of wheat bran-the mean increase in fecal weight per g/d of wheat fiber was 5.4 g. The mean increases in fecal weight per g/d of other sources of fiber were smaller in magnitude: fruit and vegetables (4.7 g), gums and mucilages (3.7 g), cellulose (3.5 g), oats (3.4 g), corn (3.3 g), legumes (2.2 g), and pectin (1.2 g). Of note, findings for the other cereal sources of ICDF were from fewer studies. Nevertheless, based on the available evidence, wheat fiber was the most effective source of fiber for increasing fecal weight.

The varying effects of different dietary fibers on fecal bulking are likely related to different underlying mechanisms of action. The effects of wheat bran fiber on stool weight are largely attributable to its high resistance to fermentation by colonic bacteria, combined with its water binding capacity (1 g of fiber binds about 3 g of water), therefore contributing to a stronger effect on increasing stool bulking compared to more easily fermented ICDF, such as those from oats and barley[14,86,87]. The resulting increased volume of fecal mass stimulates colonic movement, thereby helping to reduce transit time and increase stool frequency[14].

Since the review on fiber and bowel function conducted by Cummings[14] more than 40 interventions have been published, 30 of which were in healthy populations and therefore included in the present evaluation. The heterogeneity of the included studies did not allow for a meta-analytical approach according to PRISMA requirements. Therefore, a weighted regression analysis by sample size was conducted

as an alternative approach to achieve a quantitative estimate. Similar to the findings by Cummings[14], an increase in fecal weight was observed in the current analysis. The smaller estimated increase in fecal weight, compared to the earlier review (3.7 g/d *vs* 5.4 g/d)[14], is likely due to the weighted regression method applied in the present analysis, in which the regression equation was not forced through zero, thus influencing the slope of the regression line. Based on a visual inspection of a funnel plot on the total stool weight data (Appendix 5), publication bias is unlikely the cause of the positive intercept of the regression. Furthermore a greater number of placebo-controlled trials were included in the present analysis.

In addition, changes in other bowel function parameters, such as stool frequency, transit time, dry stool weight, and percentage water in stools were also quantitatively evaluated; studies on stool composition were too heterogeneous to allow for a quantitative approach. Provision of wheat fiber showed beneficial effects on dry stool weight and stool frequency, as well as on intestinal transit time among those with an initial transit time greater than 48 h. This arbitrary level of 48 h was used because normal stool frequency was considered to be between 1 to 2 bowel movements per day. When transit time is already optimal, *i.e.*, between 24 and 48 h, additional dietary fiber would not be expected to alter transit[88]. Adding dietary fiber that is resistant to fermentation does not increase the overall percentage of water as the amount of water bound by the fiber is similar to the average water content of fecal samples (about 75%).

Different methodologies were used in the different studies to determine transit time. First, several markers, including indigestible dye, radio-opaque markers, poly-ethylene glycol, and chromium sesquioxide, were used to estimate transit time. Secondly, transit time was calculated in different ways based on the recovery of the markers in the feces. Cummings *et al*[32] demonstrated that the mean transit time method with a single dose estimate was approximately 15% lower compared to an estimate with the 80% method. Wrick *et al*[51] examined the use of radio-opaque pellets, poly-ethylene glycol and chromium sesquioxide as markers to estimate transit time and concluded that there was no significant difference in transit time estimates between marker types. It remains possible that the different methods may yield different estimates of transit time. However, an analysis stratified by the type of methodology to estimate transit time would have lowered the power of the analysis. We concluded that a weighted regression analysis on all available data, categorized according to initial transit time with a cut off point of 48 h, provided the best estimate on the effects of ICDF on transit time.

Inadequate dietary fiber intake is increasingly a global concern, as average intakes are well below recommendations across many countries[10,11]. The International Life Sciences Institute Europe Dietary Carbohydrates Task Force summarized sex-specific dietary fiber consumption across nine European countries, in addition to the United States and Japan[10]. The resulting report found that average dietary fiber intakes were below the lower end of the World Health Organization recommendation (25-40 g/d)[89], with only a few exceptions. These findings have potentially serious health consequences beyond impaired bowel function[60,83]. Inadequate dietary fiber intakes have been associated with increased risk for type 2 diabetes, cardiovascular disease, certain cancers, weight gain, diverticular disease, obesity, and constipation[60,83]. Given the high content of dietary fiber in wheat bran (43 g compared to 21 g in rice bran and 15 g in oat bran, per 100 g[12]), wheat bran can play an important role in helping individuals increase overall dietary fiber intakes. Increasing wheat bran intake is a relatively simple dietary strategy to improve bowel function.

A notable strength of this research is the large volume of studies evaluated (n = 65), highlighting its comprehensive and inclusive nature on ICDF and bowel function. A number of parameters of bowel function that had not been quantitatively evaluated previously were examined, which is a substantial contribution to the literature. In addition, this review includes 20 years of research since the last review by Cummings[14]. Several limitations should also be considered. Due to the exhaustive and inclusive nature of this review, a large number of included interventions were uncontrolled trials, and most studies were not randomized. Therefore, observed changes in parameters for healthy bowel function cannot be fully attributed to the intervention, as the placebo effect remains possible[90]. In addition, proper quantitative evaluations of the effects of other ICDF were not feasible due to the limited available data. Future studies that examine other ICDF will provide valuable contributions to this line of research.

In summary, the current comprehensive review of interventions with ICDF on bowel function is spanning more than 90 years of research in healthy individuals. The results of the 65 included publications indicate that wheat fiber promotes healthy bowel function through improvements in total stool weight, dry stool weight, intestinal transit time, and stool frequency. Based on the large volume of available evidence, incorporating wheat fiber, primarily wheat bran fiber, into the diet can positively affect bowel function. As wheat was the only cereal for which a quantitative estimate of its effect was possible, more research on the effects of other cereals is warranted.

COMMENTS

Background

Composition, consistency, frequency, and weight of bowel movements are key indicators of intestinal and digestive health. Infrequent bowel movements and

low stool weights are very common in many countries.

Research frontiers

The effects of intact cereal dietary fibers on bowel function have not been systematically reviewed previously. Constipation and irregular bowel movements are highly prevalent in the general population but can often be averted through simple, realistic, and inexpensive changes in dietary practices such as increasing dietary fiber intakes.

Innovations and breakthroughs

This systematic review provides the first quantitative estimate of the effect of wheat fiber on multiple measures of bowel function based on the results of 90 years of research.

Applications

Findings from this comprehensive review may help gastroenterologists choose relatively inexpensive solutions in the prevention of constipation.

Peer-review

This is a useful review on the impact of specific dietary cereal fibers on bowel functions.

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Allocation of patients with liver cirrhosis and organ failure to intensive care: Systematic review and a proposal for clinical practice

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Abstract

AIM: To propose an allocation system of patients with liver cirrhosis to intensive care unit (ICU), and developed a decision tool for clinical practice.

METHODS: A systematic review of the literature was performed in PubMed, MEDLINE and EMBASE databases. The search includes studies on hospitalized patients with cirrhosis and organ failure, or acute on chronic liver failure and/or intensive care therapy.

RESULTS: The initial search identified 660 potentially relevant articles. Ultimately, five articles were selected; two cohort studies and three reviews were found eligible. The literature on this topic is scarce and no studies specifically address allocation of patients with liver cirrhosis to ICU. Throughout the literature, there is consensus that selection criteria for ICU admission should be developed and validated for this group of patients and multidisciplinary approach is mandatory. Based on current available data we developed an algorithm, to determine if a patient is candidate to intensive care if needed, based on three scoring systems: premorbid Child-Pugh Score, Model of End stage Liver Disease score and the liver specific Sequential Organ Failure Assessment score.

CONCLUSION: There are no established systems for allocation of patients with liver cirrhosis to the ICU and no evidence-based recommendations can be made.

Key words: Cirrhosis; Failure; Intensive care; Allocation; Treatment

Core tip: The literature regarding allocation of cirrhotic patients to intensive care unit (ICU) is very limited and no studies have proposed and tested any specific allocation criteria. Thus it still remains to be determined, which cirrhotic patients will benefit from intensive care treatment, and if so, when during admission they should be transferred to the ICU, and when intensive treatment is futile and should be withheld. We propose an allocation system for clinical practice, based on internationally validated scoring systems.

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INTRODUCTION

The incidence of liver cirrhosis is increasing[1,2]. In the western world liver cirrhosis is mainly related to the increasing burden of alcohol-related liver disease, non-alcoholic fatty liver disease and currently also hepatitis C, in the eastern world hepatitis B is a main cause. Patients with liver cirrhosis are prone to a progressive deterioration with the occurrence of portal hypertension and hepatic failure leading to end-stage liver disease and the development of complications. Furthermore, a large group (up to 75%) of patients are first diagnosed with liver cirrhosis, when experiencing their first episode of decompensation, and thus these patients represents a large hidden burden of the disease[3]. Patients with advanced liver cirrhosis, frequently require admission to intensive care unit (ICU)[4], mainly for various complications of advanced liver disease (*i.e.,* sepsis, variceal bleeding, hepatic encephalopathy, spontaneous bacterial peritonitis, hepato-renal syndrome). These complications are associated with high risk of organ failures and a high mortality. Patients with cirrhosis presenting with acute hepatic deterioration and progressive organ failure represent the, now well described, syndrome acute-on-chronic-liver failure (ACLF)[5,6]. The Chronic Liver Failure (CLIF) ACLF in cirrhosis (CANONIC) study investigators of the European Association for the Study of the Liver - Chronic Liver Failure Consortium (EASL-CLIF) originally introduced the concept of ACLF: "ACLF is based on the presence of three major characteristics of the syndrome; acute decompensation, organ failure [predefined by the Clif Sequential Organ Failure Assessment (SOFA) score] and high 28-d mortality rate[6]". Since ACLF affects approximately 30% of hospitalised cirrhotic patients, ACLF is the most frequent indication for admission to the ICU[4,5]. However, the mortality among patients admitted to the ICU with ACLF is high[7]. Consequently, clinical awareness on identification of cirrhotic patients at risk of developing organ failure, who will benefit from early intensive care treatment, is of great importance. The mortality among patients admitted to the ICU with ACLF ranges between 35%-93%[2,5,7]. Patients requiring mechanical ventilation are high-risk patients with poor long-term survival with a 1-year mortality rate of 89%[8]. Kavli *et al*[7] have shown that among patients with clinical or histological diagnosed alcoholic liver cirrhosis, in need for intensive care treatment, the 90-d mortality reaches levels of up to 93%, dependent on the degree of organ system failure[7]. Due to the poor prognosis the utilization of organ support in the ICU for these patients is often questioned. The treatment of patients with ACLF, not eligible for liver transplantation, is complicated and must be managed with therapies, which require a highly specialized team and close collaboration between hepatologists and intensive care specialists. There is a need for clinical tools and a proper triage to guide the physicians in decision making regarding the allocation of patients with ACLF to ICU, admitting only patients who would likely benefit from intensive care treatment[7]. In this study we aimed to perform a systematic review regarding allocation of patients with liver cirrhosis to intensive care, and to propose a decision tool for clinical practice.

MATERIALS AND METHODS

An electronic search was performed in PubMed, MEDLINE and EMBASE. Studies were included in the search irrespective of blinding, publication status, or language, and manual searches including scanning of reference lists in relevant articles and conference proceedings was also performed. All studies responding to the issue of this article were eligible for analysis, including previous reviews.

Criteria for considering studies for this review

The present systematic search includes studies on hospitalized patients with cirrhosis and organ failure or acute on CLIF and/or intensive care therapy.

Search methods for identification of studies

String 1: "liver cirrhosis" or "hepatic cirrhosis"; String 2: "liver failure" or "hepatic failure"; String 3: "intensive care" or "intensive treatment" or "intensive therapy" or "critical care".

Potentially eligible studies were listed and evaluated. After reading the titles and abstracts, clearly irrelevant trials were excluded. Afterwards relevant full text studies were evaluated, and the eligible studies

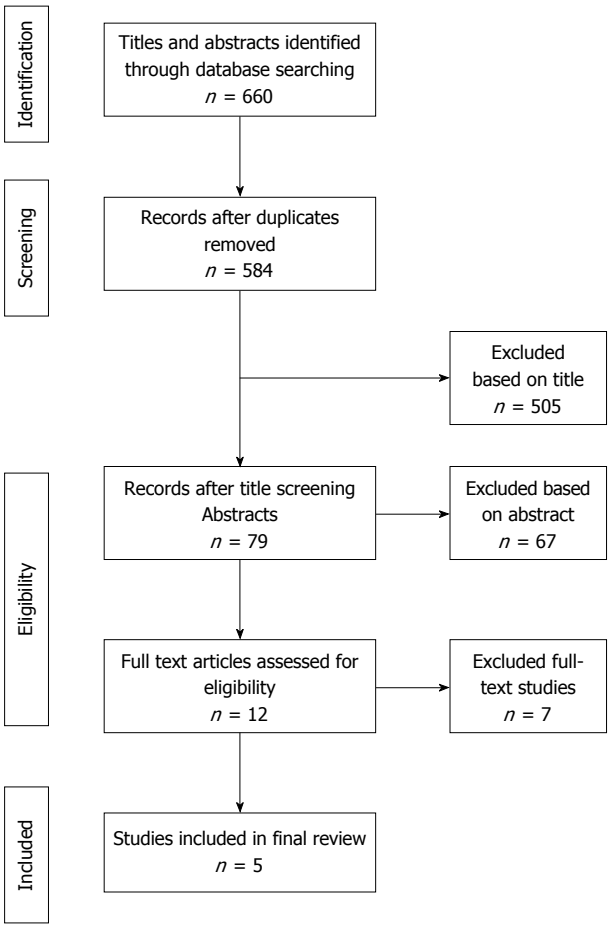

Figure 1 Flowchart of trials. Flowchart of search string.

were selected (Figure 1).

RESULTS

The initial search identified 660 articles, 402 articles were indexed in PubMed, and 258 indexed in EMBASE. After duplicates were removed 584 articles, were evaluated for relevance.

During the initial title screening process 505 articles where evaluated by title of the first author, and found not relevant for the specific topic of this article, and therby excluded from the study. Further 67 articles were excluded after reading the abstracts, leaving 12 potentially relevant articles to be evaluated in full text together with a review of references in these articles. Ultimately, five articles were selected; two cohort studies and three reviews were found in the systematic search to be eligible for the present review (Figure 1). Table 1 summarizes the main findings of the included studies.

Cohort studies

Kavli *et al*[7] performed a retrospective cohort study including 87 patients admitted to an ICU, at a university hospital with primary and secondary referrals. They found that increasing numbers of organ failures, correlate with increased mortality in critically ill patients with alcoholic liver cirrhosis. Interestingly they found that in patients with 3 or more organ failures the ICU mortality was > 90% (Table 1). Furthermore, they found the prognostic scores specific for critical illness to be superior at predicting outcome compared to the liver-specific Child-Pugh score.

Shawcross *et al*[1] performed a prospective analysis of 563 patients admitted to a liver ICU with diagnosis of cirrhosis and dysfunction of one or more extrahepatic organ(s). They assessed resource utilization and found that presence of organ failure resulted in considerable resource expenditure in patients with liver cirrhosis, but had hospital mortality of 59% as illustrated in Table 1.

Reviews

Ginès *et al*[9] performed a review with focus on the diagnostic approach and the currently recommended treatment strategy of critically ill cirrhotic patients. They report no new data, and no methods section is described. However, they describe that several studies have shown that relatively good results can be obtained in selected critically ill cirrhotic patients, and therefore reluctance to refer these patients to the ICU should be balanced. They suggest that patients with a low Model of End-stage Liver Disease (MELD score) (< 15) should be immediately considered for ICU. Contrary, in patients with end-stage cirrhosis (MELD > 30), 3 or more organ failures, and no perspective of transplantation, aggressive management is questionable.

Berry *et al*[10] performed a review within the field of cirrhotic patients and allocation to ICU. They reported no new data, and the methods section was sparsely described. They suggest that an aggressive approach to organ support is justified, and that further discussions between hepatologists and intensive care specialists are required to determine acceptable burden-to-benefit ratios for prolonged intensive care support in young alcoholic patients.

Saliba *et al*[11] performed a review within the field of cirrhotic patients and allocation to ICU. They reported no new data, and no methods section was described. However, they claimed that general ICU prognostic scores [SOFA score, Acute Physiology and Chronic Health Evaluation (APACHE) Ⅱ and SAPS Ⅱ] predicted mortality better than liver specific scores (MELD and Child-Pugh score) in cirrhotic patients, once admitted to the ICU. Furthermore, ICU and liver scores predicted outcome more precisely once they were re-evaluated at day 3 from admission. The authors stated that patients should be referred from the hepatology ward to the ICU at an earlier stage of decompensation, and a multidisciplinary approach between hepatologists, intensive care specialists, and also transplant surgeons should be mandatory.

Scoring systems

A number of score systems have been developed to assess the prognosis of critically ill cirrhotic patients,

Table 1 Included trials/studies

Author, year, location, study design	Main target of the study	Patient population	Scores	Allocation system	In hospital mortality	ICU mortality
Kavli *et al*[7], 2012, Denmark, cohort study	This study investigated the severity of organ failure, and the frequency and outcome of withholding therapy in patients with advanced alcoholic liver cirrhosis admitted to a Scandinavian ICU	87 adult patients with clinical or histological diagnosis of liver cirrhosis admitted to ICU at a University hospital in Denmark, within a 3 years period from January 2007-January 2010	APACHE Ⅱ, SAPS Ⅱ, and SOFA were better at predicting mortality than Child-Pugh score	No specific allocation system is proposed	Only ICU data	With 3 or more organ failures the ICU mortality was > 90%
Shawcross *et al*[1], 2012, United Kingdom, cohort study	The aim of this study was to prospectively study the resource allocation and cost of a large cohort of patients with cirrhosis and one or more extrahepatic organ failure(s)	563 patients were admitted to the Liver ICU at King's College Hospital, between 2000 and 2007	The median (IQR) for all patients admitted and surviving for > 8 h on day 1 (n = 548) was Child-Pugh score 12 (11-13), MELD 25 (14-34), APACHE Ⅱ 22 (16-28) and SOFA 11 (8-13)	No specific allocation system is proposed Patients with cirrhosis admitted to ICU require high levels of organ support but ICU admission is not necessarily futile	Overall hospital mortality of 59% (330/563)	256/563 (51%) patients died whilst in the Liver ICU
Ginès *et al*[9], 2012, Spain, review	This review focuses on the diagnostic approach and treatment strategies currently recommended in the critical care management of patients with cirrhosis	None	MELD and Child-Pugh scores have important limitations in the establishment of prognosis in critically ill cirrhotic patients	Encephalopatic cirrhotic patients (grade 3 or 4 hepatic encephalopathy) require ICU admission and intubation Patients with a low MELD score (< 15), should be immediately considered for ICU. Contrary, in patients with end-stage cirrhosis (MELD > 30), 3 or more organ failures, and no perspective of transplantation, aggressive management is questionable	Hospital mortality rates in patients with 1, 2 or 3 organ/system failures were 48%, 65%, and 70%, respectively	ICU and 6-mo mortality rates of 41% and 62%, respectively 59% of cirrhotic patients placed on mechanical ventilation died during their stay in the ICU
Berry *et al*[10], 2013, United Kingdom, review	This review focuses on patients with cirrhosis, especially survival analysis and prognostic models		Child-Pugh score does not perform as well as general critical illness scoring systems The MELD score performs better than the Child-Pugh score, yet the SOFA score is superior to both Child-Pugh and MELD score	No specific allocation system is proposed Early aggressive approach to organ support is justified	Greater than 60%	ICU mortality of up to 65%, rising to 90% with sepsis, if more than 1 d of respiratory support and renal support were required
Saliba *et al*[11], 2013, France, review	This review focuses on prognostic scores and admission to ICU for critically ill cirrhotic patients	None	Suggests that ICU scores (SOFA, APACHE Ⅱ, SAPS Ⅱ) predict the outcome of cirrhotic patients admitted to the ICU better than liver scores (MELD and Child-Pugh)	No specific allocation system is proposed The persistence after ICU admission of three or more organ failures and the need for three or more organ supports, may lead to consider a limitation in life sustaining treatments, as a fatal outcome is almost constant	Only ICU data	Ranges between 34%-69%

Included studies of the present review. The table describes the included studies. ICU: Intensive care unit; SOFA score: Sequential Organ Failure Assessment score; MELD: Model of End-stage Liver Disease.

Table 2 Scoring systems to predict mortality

Score	Target	Number of studies	AUCROC range (min-max)
Child-Pugh	Prognostic[1]	14	0.71-0.87
MELD	Prognostic[1]	8	0.77-0.93
RFH	Prognostic[1]	1	0.79
SOFA	Organ failure[2]	11	0.81-0.95
APACHE Ⅱ	Prognostic in ICU[3]	9	0.66-0.83
APACHE Ⅲ	Prognostic in ICU[3]	4	0.78-0.91

[1]Prognostic: score used to assess the prognosis of chronic liver disease; [2]Organ failure: score with the objective to evaluate the degree of organ failure; [3]Prognostic in ICU: severity of disease classification score. The table describes the performance of the presented scoring systems[17,19-35]. AUROC: Areal Under the Reciever Operating Curve; Range (min-max) describing the minimum and maximum reported AUC for the given score, based on published trials[17,19-35]. ICU: Intensive care unit; MELD: Model of End-stage Liver Disease; RFH: Royal Free Hospital Score; SOFA: Sequential Organ Failure Assessment score; APACHE: Acute Physiology and Chronic Health Evaluation.

Table 3 Child-Pugh score

Measure	1 point	2 points	3 points
Total billirubin (μmol/L)	< 34 (< 1.9 mg/dL)	34-50 (< 1.9-2.9 mg/dL)	> 50 (> 2.9 mg/dL)
S-Albumin (g/L)	> 35	28-35	< 28
PT INR	< 1.70	1.71-2.30	> 2.30
Ascites	None	Mild	Moderate/severe
Hepatic encephalopathy	None	Grade Ⅰ - Ⅱ	Grade Ⅲ-Ⅳ
Points	Class	One year survival	Two year survival
5-6	A	100%	85%
7-9	B	81%	57%
10-15	C	45%	35%

Child-Pugh Score and associated one- and two-year survival.

see Table 2[2,12-35]. The liver specific scores have mainly focused on the general prognosis of cirrhotic patients, whereas the general scores, such as APACHE score, and SOFA score, were developed to predict outcome in the general population admitted to the ICU[5,14,20]. Numerous organ-failure scores have been developed to evaluate the prognosis depending on the number of organ failures, however, only a limited number of score systems have been established in clinical practice. To assess severity and prognosis due to underlying liver disease before ACLF or acute hospitalization, Child-Pugh score and MELD score are the best-validated scores[14]. The World Health Organization (WHO) performance status (PS) is also useful to grade general well-being and activities of daily life.

The prognostic liver specific score named Child-Pugh score (Table 3) contains five components; grade of hepatic encephalopathy, presence of ascites, serum-bilirubin, serum-albumin, and the international normalized ratio for prothrombin time (INR)[36].

Child Pugh score

The Child-Pugh score has been well established in clinical practice for decades. It contains a limited number of variables, is simply calculated, and is easily interpreted. The score is a simple method for determining the prognosis of patients with cirrhosis[15]. However, the Child-Pugh score does not take factors such as cardiovascular, renal, and pulmonary dysfunction into account, consequently it does not offer valid information to predict mortality in cirrhotic patients who have organ failure[14]. Further, the assessment of hepatic encephalopathy and presence of ascites are subjective measures and prone to observer variation.

MELD score

The MELD score is a scoring system for assessing the severity of chronic liver disease. The MELD Score ranges from 6 (less sick) to 40 (very sick) and the formula is based on the natural logarithm of serum bilirubin, serum creatinine, the INR for prothrombin time, and information regarding dialysis[37].

The MELD Score formula: [0.957 × ln[serum creatinin] + 0.378 × ln[serum bilirubin] + 1.120 × ln[INR] + 0.643] × 10 (if hemodialysis, value for Creatinine is automatically set to 4.0).

Serum bilirubin is a blood sample that reflects the liver function. (Reference interval: 5-25 μmol/L). The INR reflects the ability of the liver to produce proteins; more specifically the clotting factors that contribute to the coagulation process (Reference interval: 0.8-1.2 is normal, higher is abnormal). Creatinine is a blood sample that reflects the function of the kidneys. (Reference interval: 60-105 μmol/L).

The MELD score is widely used to quantify end-stage liver disease in patients listed for liver transplantation[15], and has been repeatedly shown to be equivalent or even superior to the Child-Pugh score to estimate short-term survival among cirrhotic patients[14]. An additional advantage of MELD is the exclusion of subjective measures.

WHO performance score

The performance score was originally introduced in the treatment of cancer patients, as an attempt to quantify cancer patients' general well-being and activities of daily life (Table 4)[38]. The score is widely used in other medical conditions as a simple measure of general condition, functional ability and quality of life.

APACHE scores

The APACHE score is one of several ICU scoring systems. The score is a severity-of-disease classification system, based on 12 physiological parameters; 1, alveolar-arterial O_2 tension difference ($AaDO_2$) or arterial oxygen tension (PaO_2) (depending on the fraction of inspired oxygen); 2, temperature (rectal); 3, mean arterial pressure; 4, arterial pH; 5, heart rate;,

Table 4 World Health Organization performance score

Grade	WHO Performance score
0	Fully active, able to carry on all pre-disease performance without restriction
1	Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, *e.g.*, light house work, office work
2	Ambulatory and capable of all selfcare but unable to carry out any work activities. Up and about more than 50% of waking hours
3	Capable of only limited selfcare, confined to bed or chair more than 50% of waking hours
4	Completely disabled. Cannot carry on any selfcare, totally confined to bed or chair
5	Dead

WHO: World Health Organization.

6, respiratory rate; 7, sodium (serum); 8, potassium (serum); 9, Creatinine; 10,. Haematocrit; 11, white blood cell count; and 12; Glasgow Coma Scale. The score is applied within 24 h of admission to the ICU, the higher the score, the higher risk of death[15]. Compared to Child-Pugh scores it has been shown in numerous studies to be more powerful in predicting hospital mortality in patients with liver cirrhosis. Wehler *et al*[39] found an areal under the receiver operating curve (AUROC) of 0.79 for the APACHE Ⅱ score, compared to Child-Pugh with an AUROC of 0.73, other studies have found similar results[24-26,28].

SOFA Score and CLIF-SOFA

The SOFA score was developed as a simple prognostic score to evaluate the degree of organ failure in patients in the general ICU[2,29]. The score contains information on degree of failure regarding liver, kidney, brain, coagulation, circulation and lungs, graded from 0-4 (Table 5). The CLIF-SOFA score is a modified version of the original SOFA score, a newly developed scoring system exclusively for patients with end-stage liver disease, and it accommodates a lower threshold for serum creatinine, because minor increases in s-creatinine levels in cirrhotic patients indicate marked reductions in glomerular filtration rate. Furthermore, it uses the INR instead of platelets as a marker of coagulaopathy, increases the threshold for bilirubin to achieve organ failure, and uses grades of hepatic encephalopathy from the West Haven score as opposed to Glasgow Coma Scale for neurologic failure[2,6]. Organ failure in the CLIF SOFA score is defined as; liver failure: bilirubin > 205 μmol/L, kidney failure: creatinine > 177 μmol/L or requiring renal replacement therapy, neurologic failure: hepatic encephalopathy grade 3 or 4, coagulation failure: INR > 2.5, and circulation failure: requiring vasopressors to maintain adequate mean arterial pressure (MAP). The CLIF-SOFA score can be translated into a degree of ACLF[6,40] (Figure 2, Tables 5 and 6).

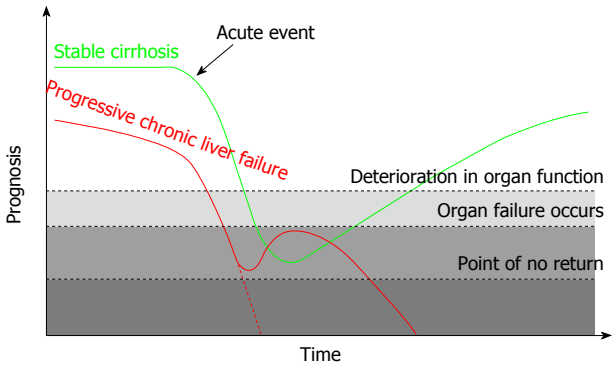

Figure 2 Acute on Chronic Liver Failure. The green line illustrates a patient with stable cirrhosis (*i.e.*, Child-Pugh class A/B) developing acute on chronic liver failure (ACLF). The red line illustrates a patient with progressive chronic liver failure (*i.e.*, a patients with Child C cirrhosis and refractory ascites) developing ACLF. The Y-axis (Prognosis) is based on Child Pugh score or MELD score and the performance status before ACLF develops and CLIF-SOFA score after ACLF. The threshold "deterioration in organ function" represents a level with *i.e.* increase in creatinine or oxygen demand without organ failure. The threshold "organ failure" is as defined in the CLIF-SOFA score and "point of no return" is a state of multiorgan failure without reversibility.

Royal Free Hospital score

The Royal Free Hospital (RFH) score is a novel prognostic model for critically ill patients with cirrhosis. Parameters included in the score are bilirubin, INR, lactate, urea, A-a gradient, and variceal bleeding. Theocharidou *et al*[18], found the RFH score to have good discriminative power, equally to the CLIF-SOFA and SOFA score, however, the score remains to be externally validated, to confirm the clinical utility.

DISCUSSION

The decision-making regarding allocation of cirrhotic patients to ICU remains a great interdisciplinary challenge in daily clinical practice. The literature is scarce and no studies specifically address this issue. There is general consensus that selection criteria for ICU admission candidates should be developed and validated for this group of patients and multidisciplinary approach is mandatory. However, all together the general approach throughout the literature is somewhat indefinable. It still remains to be determined, which cirrhotic patients will benefit from intensive care treatment, and if so, when during admission they should be transferred to the ICU, and contrary when intensive treatment is futile and should be withheld.

It has been known for more than 40 years that surgical procedures on cirrhotic patients is associated with increased risk[35], and for cirrhotic patients (child-pugh C) who undergo nontransplant surgical procedures, mortality rates of up to 76% have been reported[41]. Furthermore it is known that the mortality rate is markedly increased for emergency surgery (57%) compared to elective surgery (10%)[42]. Higher

Table 5 Chronic Liver Failure-Sequential Organ Failure Assessment score

Organ	Variabel	0	1	2	3	4
Liver	Billirubin, μmol/L (mg/dL)	< 20 μmol/L (-1.1)	⩾ 20 to < 34 μmol/L (⩾ 1.1 to < 1.9)	⩾ 34 to < 103 μmol/L (⩾ 1.9 to < 6.0)	⩾ 103 to < 205 μmol/L (⩾ 6.0 to < 11.9)	> 205 μmol/L (> 11.9)[1]
Kidney	Creatinine, μmol/L (mg/dL)	< 106 μmol/L (< 1.2)	⩾ 106 to < 177 μmol/L (⩾ 1.2 to < 2.0)	⩾ 177 to < 309 μmol/L (⩾ 1.2 to < 3.5)[1]	⩾ 309 to < 442 μmol/L (⩾ 3.5 to < 5)[1] Or renal replacement therapy[1]	> 442 μmol/L (> 5.0)[1]
CNS	HE grade	None	I	II	III[1]	IV[1]
Coagulation	INR	< 1.1	⩾ 1.1 to 1.25	⩾ 1.25 to < 1.5	⩾ 1.5 to < 2.5	⩾ 2.5 or platlets < 20[1]
Circulation	MAP (mmHg)	⩾ 70	< 70	Dopamine ⩽ 5[1] Dobutamine Terlipressin[1]	Dopamine > 5[1] Epinephrine ⩽ 0.1[1] Norepinephrine ⩽ 0.1[1]	Dopamine > 15 Epinephrine > 0.1 Norepinephrine > 0.1[1]
Lungs	PaO_2/FiO_2	> 400	> 300 to ⩽ 400	> 200 to ⩽ 300	> 100 to ⩽ 200[1]	⩽ 100[1]
	SpO_2/FiO_2	> 512	> 357 to ⩽ 512	> 214 to < 357	> 89 to ⩽ 214[1]	⩽ 89[1]

[1]Indicates the limit for the definition of organ failure. HE: Hepatic encephalopathy; MAP: Mean arterial pressure; PaO_2: Arterial oxygen tension; FiO_2: Fraction of inspired oxygen; SpO_2: Peripheral capillary oxygen saturation.

Table 6 Degree of Acute on Chronic Liver Failure and the associated mortality

ACLF	Numbers of organ failure	28-d mortality	90-d mortality
0	0 or 1 (/kidney)	4.7%	15.0%
1	1 (no kidney dysfunction)	22.1%	40.7%
2	2	32.0%	52.3%
3	⩾ 3	76.7%	79.1%

ACLF: Acute on Chronic Liver Failure.

risk of infection, coagulopathy and malnutrition may be important factors[43-47]. In addition many cirrhotic patients decompensate in the postoperative phase and develop hepatic encephalopathy, ascites, hepato-renal syndrome or infections[44,45,47,48]. Reduced perfusion of the liver and altered response to anaesthetics and other medications, may also be contributing factors[49,50]. It is therefore essential to assess the severity of the underlying liver disease and to perform preoperative optimisation, including treatment of coagulopathy, ascites, portal hypertension, malnutrition, any substance abuse problem and infections.

Based on current available data[5,6], we have developed an algorithm based on three scoring systems: Child-Pugh Score, MELD score and CLIF-SOFA score. In Figure 3 we suggest criteria to determine if a patient is a candidate to intensive care if needed. However, the algorithm remains to be validated in clinical practice.

The exact time point when patients should be referred to intensive care may be difficult to identify and will likely depend on local clinical setup, *i.e.,* access to intermediate care. However, the following general consideration can be used. Dialogue between physicians from the referring ward and ICU is mandatory and should be initiated at an early stage to assess if the patient is candidate to intensive care if needed and to ensure detection and treatment of organ failures as early as possible.

Close monitoring of progressive organ dysfunction in the ward is essential. Sometimes it may also be rational to transfer patients to the intermediate care/ICU to prevent occurrence of organ failure by close monitoring. Referral criteria should include the following: (1) Comatose patients not able to protect airways; (2) Respiratory failure with need of mechanical ventilation; (3) Circulatory failure with systolic blood pressure < 90 mmHg or low mean arterial pressure (MAP < 60 mmHg) despite adequate fluid supplementation and increasing lactate or increasing creatinine or reduced urine output; (4) necessity of vasopressors; and (5) septic shock or multi-organ failures.

To help the daily registration of important values in patients with liver cirrhosis, we have developed a specific computer software program First Chronic Liver Allocation Scoring System (First-CLASS).

First-CLASS features an extensive data-gathering

Intensive care candidate	Premorbid Child-Pugh A/B (5-9 points) or MELD score < 15 and ACLF grade ⩽ 2 with the need for intensive therapy, *e.g.,* HE > 2 or respiratory insufficiency
Possible intensive care candidate, eligibility should be considered and discussed with ICU	Premorbid Child-Pugh A/B (5-9 points) or MELD score < 15 and ACLF grade ⩾ 3 or Premorbid Child-Pugh C (10-15 points) or MELD score 16-29 and ACLF ⩽ 2 If continuous organ failure is present after 3 d of complete intensive care treatment, the outcome is questionable
Unlikely to benefit from intensive care therapy Special cases can be disussed, *e.g.,* patients that are candidate for liver transplantation	Premorbid Child-Pugh C (10-15 points) or MELD score > 30 and ACLF grade ⩾ 3

Figure 3 Proposal of a clinical system to identify candidates for intensive care if indicated. Algorithm based on three scoring systems: Child-Pugh Score, Model of End stage Liver Disease score (MELD) and Sequential Organ Failure Assessment score (CLIF-SOFA). ACLF: Acute on chronic liver failure; ICU: Intensive care unit.

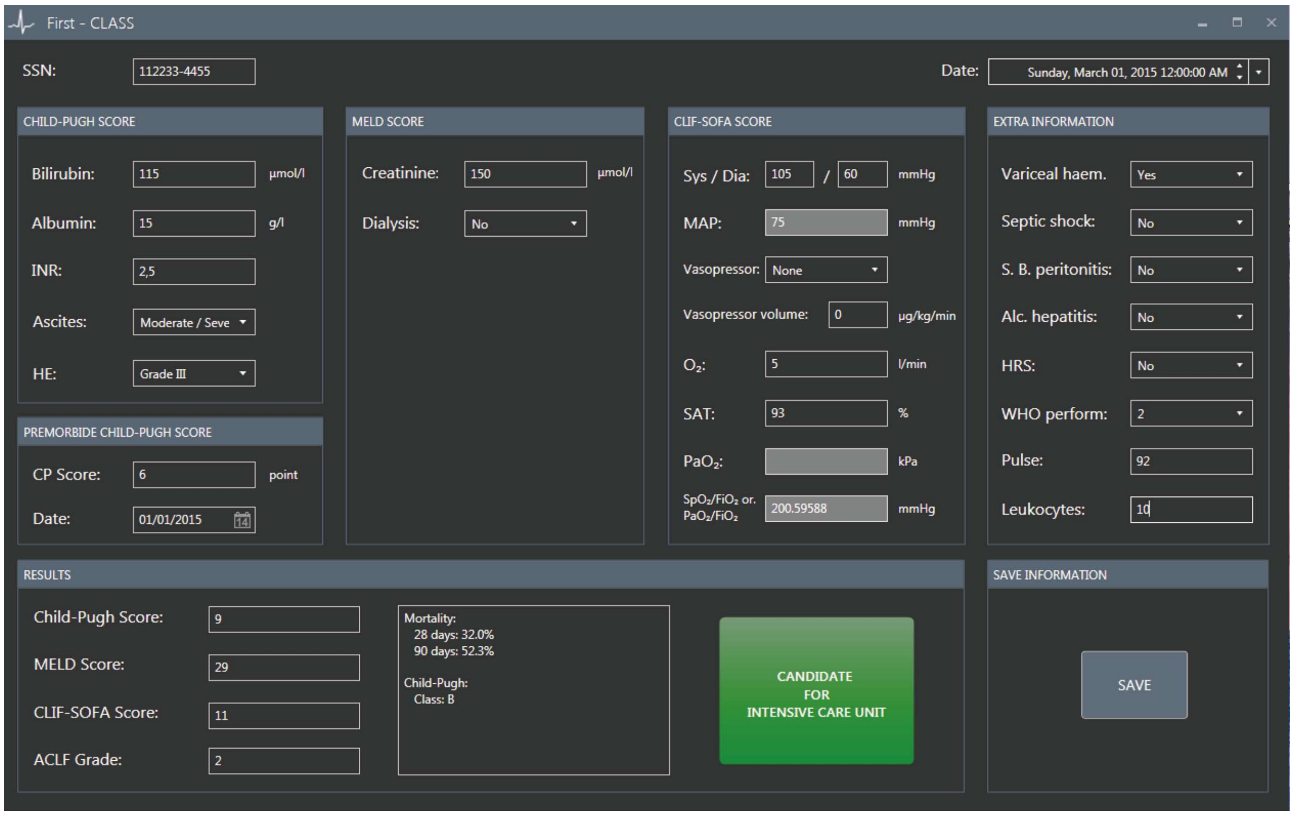

Figure 4 First Chronic Liver Allocation Scoring system. Danish civil registration number, a unique 10-digit personal identification number assigned to every Danish citizen at birth since 1968. HE: Hepatic encephalopathy; CP Score: Child Pugh Score; MAP: Mean arterial pressure; SAT: Oxygen saturation (%); Varice Haemorr: Variceal haemorrhage; S.B. Peritonitis: Spontaneous bacterial peritonitis; Alko. Hepatitis: Alcoholic hepatitis; HRS: Hepato renal syndrome; WHO Perform: WHO performance status; ACLF grade: Acute on chronic liver failure grade; First-CLASS: First Chronic Liver Allocation Scoring System; SSN: Social Security Number.

tool, with a user-friendly graphical interface, for the purpose of scoring acute on CLIF patients. An immediate score of the patient is calculated using the described algorithm, and it will be presented to the user. All data is saved to a database, which can be easily retrieved for both research and evaluation of specific patients. First-CLASS provides a program for viewing the data that has been collected in a meaningful way, such as using graphs for visualization of results in the given admission period. Furthermore the First-CLASS-Viewer program allows for exporting the data of patients to be used directly for statistical analysis (Figure 4).

In conclusion, we found that the literature regarding allocation of cirrhotic patients to ICU is very limited and no studies have proposed and tested any specific allocation criteria. Thus it still remains to be determined, which cirrhotic patients will benefit from intensive care treatment, and if so, when during admission they should be transferred to the ICU, and when intensive treatment is futile and should be withheld. Based on indirect evidence we have proposed a scoring system that can help identify which patients are candidates or not to ICU therapy, however the system needs validation.

COMMENTS

Background

Patients with liver cirrhosis are prone to a progressive disease course with the occurrence of portal hypertension and hepatic failure leading to end-stage disease and development of complications. Cirrhosis and organ failure is associated with a high mortality. Rational and timely allocation to an intensive care unit (ICU) is a challenge of clinical importance.

Research frontiers

The exact time point when patients should be referred to intensive care may be difficult to identify and will likely depend on local clinical setup, *i.e.*, access to intermediate care. Close monitoring of progressive organ dysfunction in the ward is essential. Sometimes it may also be rational to transfer patients to the intermediate care/ICU to prevent occurrence of organ failure by close monitoring. Referral criteria should include the following: (1) Comatose patients not able to protect airways; (2) Respiratory failure with need of mechanical ventilation; (3) Circulatory failure with systolic blood pressure < 90 mmHg or low mean arterial pressure (MAP < 60 mmHg) despite adequate fluid supplementation and increasing lactate or increasing creatinine or reduced urine output; (4) necessity of vasopressors; and (5) septic shock or multi-organ failures.

Innovations and breakthroughs

To help the daily monitoring and registration of organfunctions in patients with liver cirrhosis, the authors have developed computer software program First Chronic Liver Allocation Scoring System (First-CLASS). First-CLASS features an extensive data-gathering tool, with a user-friendly graphical interface, for the purpose of scoring acute on chronic liver failure (CLIF) patients. An immediate

score of the patient is calculated using the described algorithm, and it will be presented to the user.

Applications

It still remains to be determined, which cirrhotic patients will benefit from intensive care treatment, and if so, when during admission they should be transferred to the ICU, and when intensive treatment is futile and should be withheld. Based on indirect evidence, the authors have proposed a scoring system that can help identify which patients are candidates or not to ICU therapy, however the system needs validation.

Terminology

Patients with cirrhosis presenting with acute hepatic deterioration and progressive organ failure represent the, now well described, syndrome Acute-on-Chronic-Liver Failure (ACLF). The CLIF ACLF in cirrhosis study investigators of the European Association for the Study of the Liver - Chronic Liver Failure Consortium originally introduced the concept of ACLF: "ACLF is based on the presence of three major characteristics of the syndrome; acute decompensation, organ failure (predefined by the CLIF SOFA score) and high 28-d mortality rate".

Peer-review

In this manuscript "Allocation of patients with liver cirrhosis and organ failure to intensive care: Systematic review and a proposal for clinical practice", the authors aimed to perform a systematic review regarding allocation of patients with liver cirrhosis to ICU. They also proposed a scoring system to help identify the patients as candidates for ICU therapy. The subject matter of current study is of interest. This review may help clinicians chose the more suitable treatments.

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CASE REPORT

Laparoscopic transhiatal approach for resection of an adenocarcinoma in long-segment Barrett's esophagus

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Author contributions: Shiozaki A designed the research and wrote the manuscript; Shiozaki A, Fujiwara H and Konishi H performed the operation; Kinoshita O analyzed the clinicopathological data; Kosuga T, Morimura R, Murayama Y, Komatsu S, Kuriu Y, Ikoma H, Nakanishi M, Ichikawa D, Okamoto K and Sakakura C looked after the patient; and Otsuji E helped edit the manuscript.

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Informed consent statement: The patient provided informed written consent prior to study enrollment.

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Abstract

Barrett's esophagus (BE) is a precursor of esophageal adenocarcinoma and is associated with gastroesophageal reflux disease, which is often preceded by a hiatal hernia. We describe a case of esophageal adenocarcinoma arising in long-segment BE (LSBE) associated with a hiatal hernia that was successfully treated with a laparoscopic transhiatal approach (LTHA) without thoracotomy. The patient was a 42-year-old male who had previously undergone laryngectomy and tracheal separation to avoid repeated aspiration pneumonitis. An ulcerative lesion was found in a hiatal hernia by endoscopy and superficial esophageal cancer was also detected in the lower thoracic esophagus. The histopathological diagnosis of biopsy samples from both lesions was adenocarcinoma. There were difficulties with the thoracic approach because the patient had severe kyphosis and muscular contractures from cerebral palsy. Therefore, we performed subtotal esophagectomy by LTHA without thoracotomy. Using hand-assisted laparoscopic surgery, the esophageal hiatus was divided and carbon dioxide was introduced into the mediastinum. A hernial sac was identified on the cranial side of the right crus of the diaphragm and carefully separated from the surrounding tissues. Abruption of the thoracic esophagus was performed up to the level of the

arch of the azygos vein *via* LTHA. A cervical incision was made in the left side of the permanent tracheal stoma, the cervical esophagus was divided, and gastric tube reconstruction was performed *via* a posterior mediastinal route. The operative time was 175 min, and there was 61 mL of intra-operative bleeding. A histopathological examination revealed superficial adenocarcinoma in LSBE. Our surgical procedure provided a good surgical view and can be safely applied to patients with a hiatal hernia and kyphosis.

Key words: Laparoscopic transhiatal approach; Barrett's esophageal carcinoma; Hiatal hernia

Core tip: This report describes a case of esophageal adenocarcinoma arising in long-segment Barrett's esophagus associated with a hiatal hernia that was successfully treated with a laparoscopic transhiatal approach without thoracotomy. This surgical procedure provided a good surgical view and can be safely applied to patients with a hiatal hernia and kyphosis.

Shiozaki A, Fujiwara H, Konishi H, Kinoshita O, Kosuga T, Morimura R, Murayama Y, Komatsu S, Kuriu Y, Ikoma H, Nakanishi M, Ichikawa D, Okamoto K, Sakakura C, Otsuji E. Laparoscopic transhiatal approach for resection of an adenocarcinoma in long-segment Barrett's esophagus. *World J Gastroenterol* 2015; 21(29): 8974-8980 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i29/8974.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i29.8974

INTRODUCTION

Barrett's esophagus (BE) is a pathological phenomenon in which the normal squamous epithelium is replaced by a specialized or intestinal columnar epithelium[1]. BE is classified as either short-segment BE (SSBE) or long-segment BE (LSBE) according to the length of the metaplastic changes observed by endoscopic examinations[2]. In some cases, BE is accompanied by esophageal adenocarcinoma[3,4]. On the other hand, a minimally invasive approach for esophageal cancer has recently been suggested to avoid a long hospital stay. In 2009, we started performing esophagectomy with a laparoscopic transhiatal approach (LTHA) on patients with esophageal cancer[5-7]. In this method, carbon dioxide is introduced into the mediastinum from the abdominal side, and middle and lower mediastinal operations can be performed *via* a transhiatal approach. The main advantage of this method is that it provides a good surgical view of the mediastinum and improves the quality of mediastinal surgery, reducing surgical stress.

We present a case of esophageal adenocarcinoma arising in long-segment Barrett's esophagus and associated with a hiatal hernia that was successfully treated by applying LTHA.

CASE REPORT

Patient

The patient was a 42-year-old man. He had cerebral palsy from birth, and laryngectomy and tracheal separation were performed to avoid repeated aspiration pneumonitis when he was 36 years old. He presented with anorexia, anemia, and black stools. An ulcerative lesion was found by endoscopy in a hiatal hernia 30 cm from an incisor (Figure 1A), and 0-IIc type esophageal cancer was also detected 25 cm from an incisor (Figure 1B)[8,9]. The histopathological diagnosis of biopsy samples from both lesions was adenocarcinoma. Endoscopic marking with metal clips was performed near both lesions. Computed tomography (CT) revealed a sliding hiatal hernia. Metal clips were detected without wall thickening of the esophagus, lymphadenopathy, or metastasis (Figure 1C-F). Because the patient had severe kyphosis and muscular contractures caused by cerebral palsy, there were difficulties with maintaining the left lateral-decubitus position. Furthermore, single lung ventilation from the permanent tracheal stoma appeared to be technically challenging; therefore, a thoracic approach seemed to be unsuitable for him. Instead, we performed subtotal esophagectomy by LTHA without thoracotomy.

Surgical technique

The patient was placed in the supine position on the operating table. An upper abdominal incision (70 mm) was made, and a Lap Disc (regular) (Ethicon, Cincinnati, OH) was placed. Three 12-mm ports were produced, one in each flank and one in the left hypochondrium, and one 5-mm port for a flexible laparoscope was inserted into the lower abdomen, as previously described[5-7]. Carbon dioxide was introduced into the intraabdominal space, and the pneumoperitoneum pressure was controlled at 10 mmHg. The operator lifted up the stomach with the left hand, and the greater omentum, left gastroepiploic vessels, and gastrosplenic ligament were then divided using an EnSeal device (45-cm shaft length, Ethicon). The esophageal hiatus was then opened, and carbon dioxide was introduced into the mediastinum (Figure 2A). The assistant inserted an ENDO RETRACT (Autosuture Norwalk, CT) and blunt tip dissector through the ports on the left side, and the working space in the mediastinum was secured with these two devices and 10 mmHg of pneumomediastinum pressure, as previously described[5-7].

Dissection of the anterior and left side of the distal esophagus was performed up to the level of the tracheal bifurcation. The adventitia of the thoracic

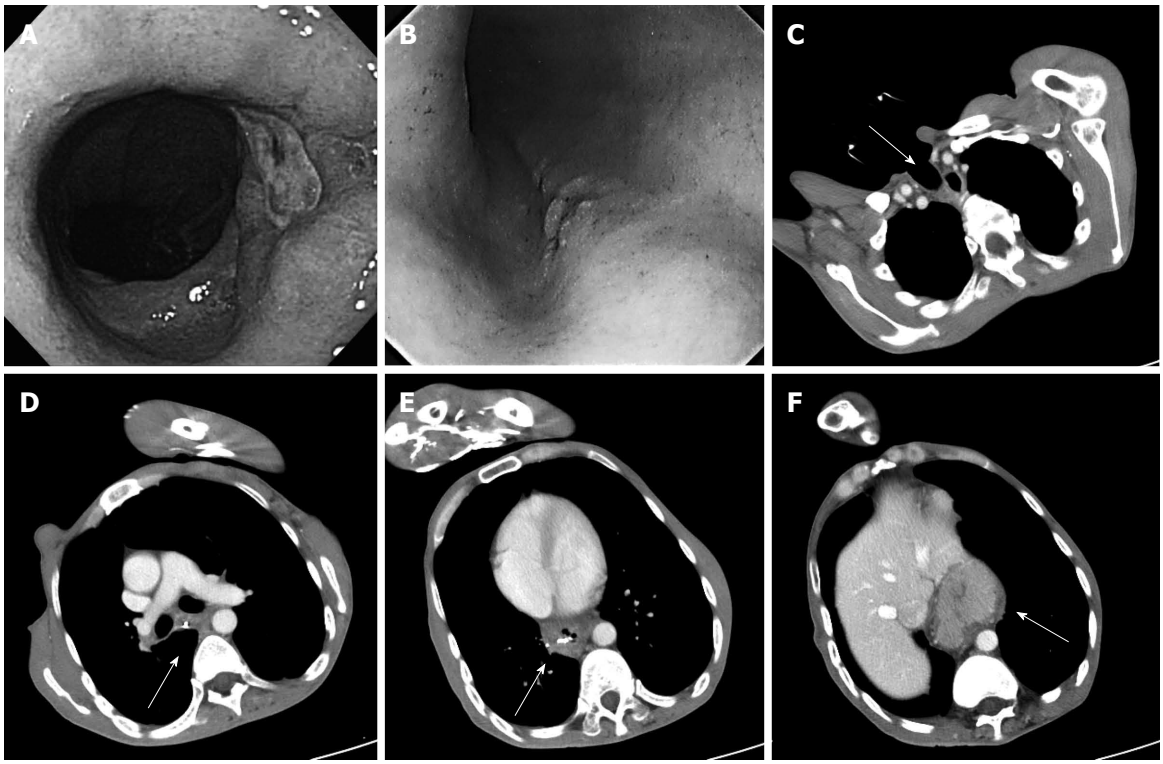

Figure 1 Endoscopy findings (A, B) and computed tomography findings (C-F). A: An ulcerative lesion was found in a hiatal hernia 30 cm from an incisor; B: 0-IIc type esophageal cancer was detected 25 cm from an incisor; There was severe deformity of the trunk caused by kyphosis and muscular contractures (C-F); C: The arrow points to the permanent tracheal stoma; D: The arrow points to the metal clip near the 0-Ⅱc type esophageal cancer; E: The arrow points to the metal clip near the ulcerative lesion in hiatal hernia; F: The arrow points to the sliding hiatal hernia.

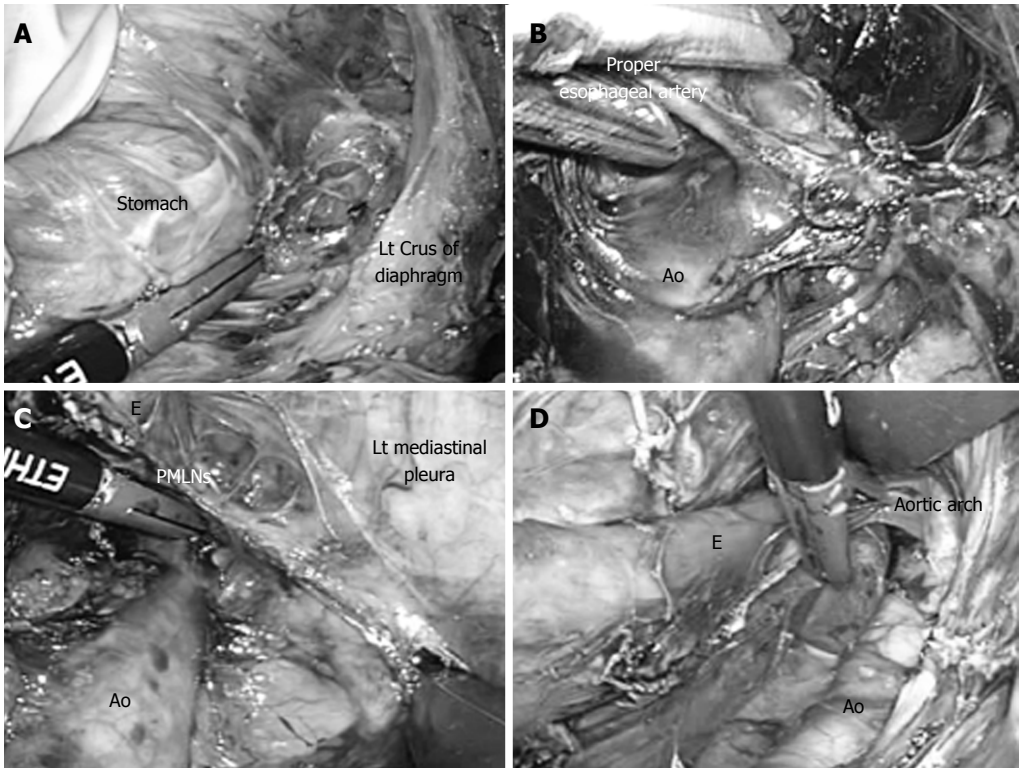

Figure 2 Surgical technique. A: The esophageal hiatus was divided, and carbon dioxide was introduced into the mediastinum; B: Dissection of the anterior plane of the thoracic aorta was extended to the cranial side, and the root of the proper esophageal artery was confirmed under a magnified videoscopic view; C: While lifting the posterior mediastinal lymph nodes like a membrane, they were cut along the border of the left mediastinal pleura; D: This incision was extended to the left pulmonary hilum and aortic arch. Ao: Thoracic aorta; E: Esophagus; Lt: Left; PMLNs: Posterior mediastinal lymph nodes.

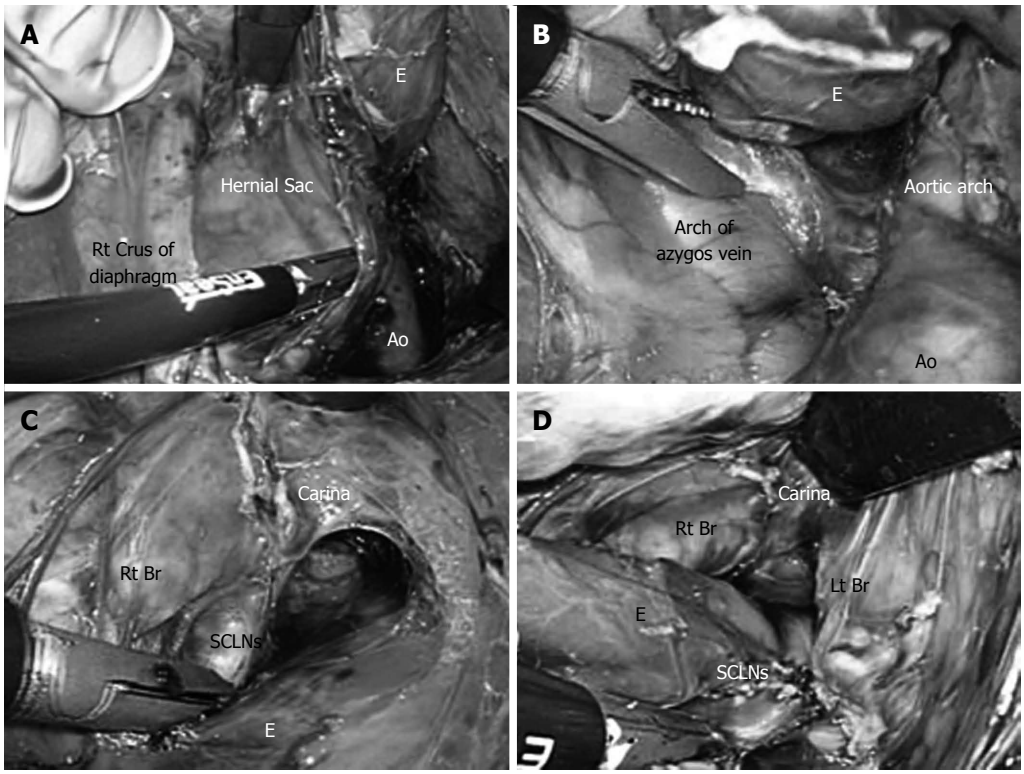

Figure 3 Thoracic esophagus was completely detached from the surrounding tissue. A: A hernial sac was identified on the cranial side of the right crus of the diaphragm; B: Dissection of the posterior and right sides of the esophagus was performed to the level of the arch of the azygos vein; C: While lifting the right mediastinal pleura like a membrane, an incision was made and extended to the right pulmonary hilum, and the lymph nodes were resected from the right main bronchus and carina; D: Intraoperative view after dissection of the subcarinal lymph nodes. Ao: Thoracic aorta; E: Esophagus; Lt: Left; Rt: Right; Br: Bronchus; SCLNs: Subcarinal lymph nodes.

aorta was then exposed at the level of the crus of the diaphragm, and we dissected the anterior side of the thoracic aorta to the cranial side. The root of the proper esophageal artery was confirmed (Figure 2B) and divided using the EnSeal device. After these procedures, both the anterior and posterior sides of the posterior mediastinal lymph nodes were dissected. While lifting these lymph nodes like a membrane, we cut them along the border of the left mediastinal pleura (Figure 2C)[6]. This incision was extended to the left pulmonary hilum and aortic arch (Figure 2D).

After the left gastric vessels were clipped and divided, the lymph nodes along the left gastric artery were dissected. Dissection of the posterior and right sides of the distal esophagus was finally performed. A hernial sac was identified on the cranial side of the right crus of the diaphragm (Figure 3A). An incision was made in the right mediastinal pleura and extended to the lower margin of the arch of the azygos vein (Figure 3B). As the left side, while lifting the right mediastinal pleura like a membrane, an incision was made and extended to the right pulmonary hilum. The lymph nodes were resected from the right main bronchus and carina (Figure 3C). In this manner, the subcarinal lymph nodes were dissected (Figure 3D)[7]. In this way, the thoracic esophagus was completely detached from the surrounding tissue.

A cervical incision was made in the left side of the permanent tracheal stoma. Cervical and upper mediastinal lymph node dissection was performed. After the cervical esophagus was divided, the esophagus was extracted from the abdominal side. After a gastric tube was created, it was pulled up *via* a posterior mediastinal route, and esophagogastric anastomosis was performed by hand from a cervical approach.

The operative time was 175 min, and there was 61 mL of intra-operative bleeding.

Postoperative course

A minor anastomotic leakage was detected on the 11th day after surgery. This leakage healed by drainage treatment. The patient was discharged 51 days after the operation. Gross and histopathological examinations revealed 0-Ⅱc type superficial adenocarcinoma (tub1 >> tub2, 72 mm × 56 mm in size, pT1a-SMM, and no lymph node metastases) of the middle and lower thoracic esophagus in LSBE (Figures 4 and 5)[8,9]. The cancer lesion was surrounded by mucosa, including a columnar epithelium that continued to the stomach (Figure 5A and B). Duplication of the muscularis mucosae was identified (Figure 5C). Although an UI-IVs type ulcer (17 mm × 17 mm in size) was identified on the distal side of the tumor, cancer was not found in this ulcerative lesion (Figure 4B).

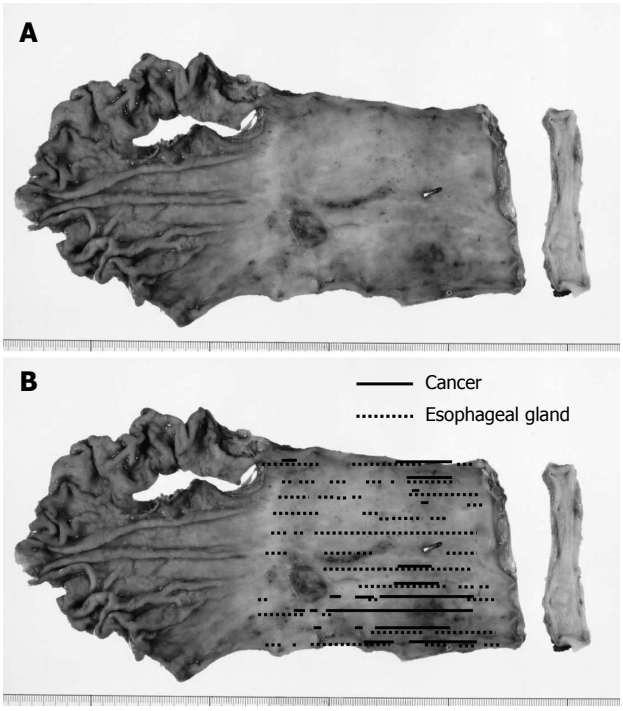

Figure 4 Resected specimen. A: A resected specimen revealed a 0-Ⅱc type tumor (72 mm × 56 mm in size) in the middle and lower thoracic esophagus in long-segment Barrett's esophagus. A metal clip was identified near the tumor; B: Mapping according to the histopathological examination. Although an UI-IVs type ulcer (17 mm × 17 mm in size) was identified on the distal side of the tumor, cancer was not found in this lesion.

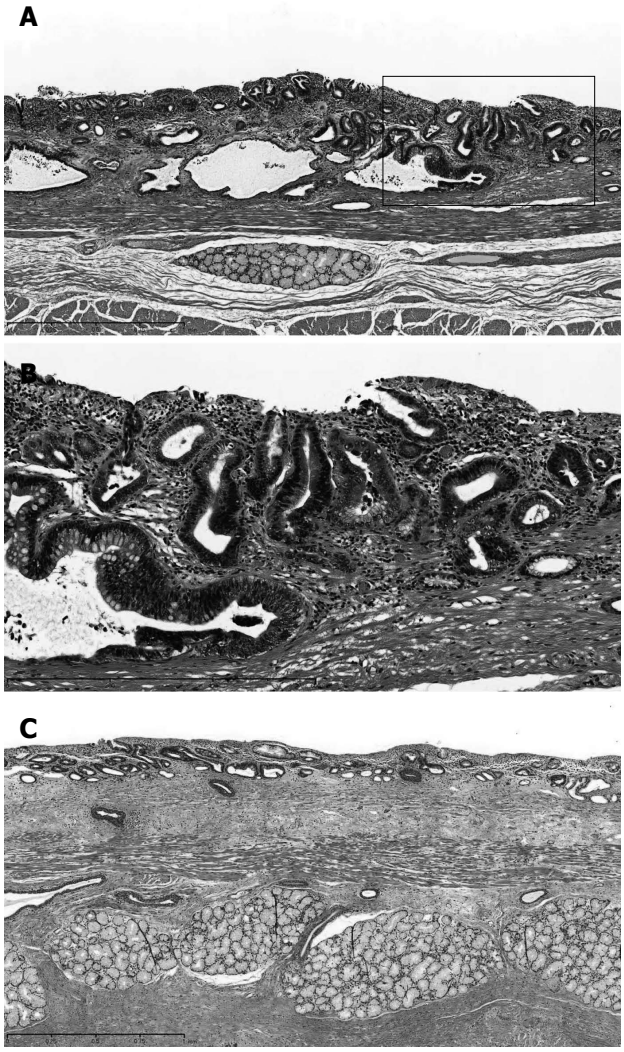

Figure 5 Histopathological examination. A: A histopathological examination revealed superficial adenocarcinoma (pT1a-SMM). The cancer lesion was surrounded by mucosa, including a columnar epithelium that continued to the stomach; B: Part of the small frame in (A) was magnified and shown; C: Duplication of the muscularis mucosae was identified in long-segment Barrett's esophagus. Hematoxylin-eosin staining; magnification, × 100 (A), × 150 (B) or × 50 (C).

DISCUSSION

BE is commonly defined as replacement of the esophageal squamous epithelium in the lower esophagus with a metaplastic simple columnar epithelium[1]. BE is associated with the presence of gastroesophageal reflux disease (GERD), and the reported prevalence of BE is between 3% and 15% among patients with GERD[10,11]. The clinical significance of BE is its association with an increased risk of developing esophageal adenocarcinoma[3], and patients with LSBE are at the highest risk of malignancy[4].

The presence of a hiatal hernia has also been associated with an increased risk of BE. The most common type is Type Ⅰ, or sliding hernia, in which the lower esophageal sphincter and a portion of the gastric cardia herniate upwards due to a widening of the muscular hiatal aperture and circumferential laxity of the phrenoesophageal membrane[12,13]. The presence of a hiatal hernia results in an anatomical impairment in the esophagogastric junction, which results in reflux of gastric material into the esophagus. Individual studies have shown a higher prevalence of hiatal hernia in BE patients than in non-BE GERD patients[14,15]. Furthermore, a recent meta-analysis revealed that the strongest relationship is between hiatal hernia and LSBE[16]. In addition, the short esophagus is most commonly from the chronic inflammation that accompanies long-standing GERD[17]. The frequency of short esophagus was previously estimated as approximately 10% of patients undergoing antireflux surgery[18]. In the present case, esophageal adenocarcinoma was identified in LSBE. Although short esophagus could not be diagnosed preoperatively because of difficulties associated with upper gastrointestinal radiography, the presence of a hiatal hernia and short esophagus were considered to be associated with pathogenesis.

In the present case, we encountered several difficulties when selecting the appropriate surgical approach. In this case, thoracic procedures performed *via* right thoracotomy would likely be difficult because of severe kyphosis. Furthermore, single lung ventilation from the permanent tracheal stoma also appeared to be technically challenging. Therefore, we decided to perform subtotal esophagectomy by LTHA without

thoracotomy. In addition, the permanent tracheal stoma restricted the selection of a reconstruction route. The presence of the permanent tracheal stoma made it difficult to use a subcutaneous or anterior mediastinal route. Therefore, we used a posterior mediastinal route in the present case.

In 2009, we started performing subtotal esophagectomy by LTHA on patients with esophageal cancer[5-7]. By November 2014, 182 patients with esophageal cancer had undergone LTHA during various esophageal surgical procedures. Our procedure has facilitated middle and lower mediastinal lymph node dissection *via* a transhiatal approach[6,7]. Because upper mediastinal lymph nodes can be dissected from a cervical approach, we do not perform thoracic surgery in preoperative high-risk patients, including the present case, to reduce surgical invasiveness[7]. The present results suggest that our surgical procedure, esophagectomy by LTHA without thoracotomy, can be safely applied in patients with a hiatal hernia, short esophagus, and severe kyphosis.

In conclusion, we described a case of esophageal adenocarcinoma arising in LSBE associated with a hiatal hernia that was successfully treated using LTHA. Our surgical procedure provides a good surgical view, improves the quality of mediastinal surgery, reduces surgical stress, and can be safely applied to patients with a hiatal hernia and kyphosis.

COMMENTS

Case characteristics

A 42-year-old male with a history of tracheal separation to avoid repeated aspiration pneumonitis presented with anorexia, anemia, and black stools.

Clinical diagnosis

An ulcerative lesion was found in a hiatal hernia by endoscopy, and superficial esophageal cancer was also detected in the lower thoracic esophagus.

Differential diagnosis

Malignant tumor (adenocarcinoma and squamous cell carcinoma) and benign disorder (ulcer, hiatal hernia and Barrett's esophagus).

Laboratory diagnosis

The patient had no remarkable findings for the laboratory tests or tumor markers (SCC, CEA, CA19-9 and Cyfra).

Imaging diagnosis

A computed tomography scan showed a sliding hiatal hernia without wall thickening of the esophagus, lymphadenopathy, or metastasis.

Pathological diagnosis

The histopathological diagnosis of biopsy samples from both an ulcer lesion and superficial esophageal cancer was adenocarcinoma.

Treatment

Subtotal esophagectomy by a laparoscopic transhiatal approach without thoracotomy was performed, and a histopathological examination revealed superficial adenocarcinoma in long-segment Barrett's esophagus.

Related reports

Barrett's esophagus is a precursor of esophageal adenocarcinoma and is associated with gastroesophageal reflux disease, which is often preceded by a hiatal hernia.

Term explanation

In the laparoscopic transhiatal approach method, carbon dioxide is introduced into the mediastinum from the abdominal side, and middle and lower mediastinal operations can be performed *via* a transhiatal approach.

Experiences and lessons

This surgical procedure provided a good surgical view and can be safely applied to patients with a hiatal hernia and kyphosis.

Peer-review

In this article, the authors reported on a patient who was successfully treated by a laparoscopic transhiatal approach. The authors' actions were novel and commendable when they encountered several difficulties in selecting the surgical approach.

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CASE REPORT

Wilson disease with hepatic presentation in an eight-month-old boy

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Author contributions: Wang JS conceived the study, treated and followed up the index patient, revised the manuscript, and approved the submission of the final draft; Abuduxikuer K wrote the manuscript, retrieved relevant information from patient files, contacted the family for further information, determined the nature of genetic mutations by consulting genetic databases, and submitted the approved draft; Li LT, Qiu YL and Wang NL collected patient files, contributed to writing the manuscript, and conducted genetic analysis.

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Abstract

Wilson disease is an autosomal recessive disorder of copper metabolism that can cause fatal neurological and hepatic disease if not diagnosed and treated. The youngest child with normal liver function reported so far is an 8-mo-old Japanese boy with low ceruloplasmin levels, and the youngest child with elevated amino-transferase ever reported so far is a 9-mo-old Korean boy with confirmed by genetic testing. Here we report an 8-mo-old Chinese boy presented with elevated liver enzymes, and low serum ceruloplasmin level. Genetic analysis of *ATP7B* gene detected two heterozygous disease causing mutations (c.2621C>T/p.A874V and c.3809A>G/p.N1270S), and parental origins were determined. Persistent elevation of serum amino-transferase in this infant was normalized after zinc therapy. To our best knowledge, this is the youngest patient with elevated liver enzymes ever reported worldwide. We hope that this will raise awareness among pediatricians, leading to earlier diagnosis, timely treatment, and better clinical outcome.

Key words: Wilson disease; Infant; Hepatic presentation; *ATP7B*; Copper; Zinc

Core tip: Wilson disease is rarely diagnosed during

infancy. The youngest child with normal liver function reported so far is an 8-mo-old Japanese boy, and the youngest child with liver function abnormality is a 9-mo-old Korean boy. Here we report an 8-mo-old Chinese boy presented with elevated liver enzymes, and low serum ceruloplasmin level. Diagnosis of Wilson disease was confirmed with *ATP7B* gene sequencing of the index case, and parental origins of disease causing mutations were outlined. To our best knowledge, this is the youngest patient with elevated liver enzymes ever reported worldwide.

Abuduxikuer K, Li LT, Qiu YL, Wang NL, Wang JS. Wilson disease with hepatic presentation in an eight-month-old boy. *World J Gastroenterol* 2015; 21(29): 8981-8984 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i29/8981.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i29.8981

INTRODUCTION

Wilson disease (WD) is an autosomal recessive disorder of copper metabolism caused by *ATP7B* gene mutation. It can cause fatal damage to the liver and brain tissues if not diagnosed and treated earlier. Age at disease onset or appearance of clinical symptoms can vary markedly among patients[1]. The youngest child with normal liver function reported so far is an 8-mo-old Japanese boy with a low ceruloplasmin level[2]. In terms of liver function abnormality, the youngest child ever reported so far is a 9-mo-old Korean boy with elevated aminotransferase confirmed by genetic testing[3]. Here we report an 8-mo-old Chinese boy who presented with elevated liver enzymes, and the diagnosis of WD was confirmed after *ATP7B* gene sequencing.

CASE REPORT

This boy first discovered to have abnormal liver function after routine blood testing during a diarrhea admission at 8-mo of age. After persistent elevation of serum alanine aminotransferase (ALT), and aspartate aminotransferase (AST) levels, WD was suspected. Serum ceruloplasmin level was found to be extremely low, and disease causing mutations were found on ATP7B gene sequencing. The patient was given oral zinc therapy with 10 mg of elemental zinc 3 times daily. At 14 mo of age, liver function significantly improved but ALT and AST levels were still above normal range. Persistent normalization was achieved after zinc gluconate dosage was increased to 20 mg/3-times-a-day (Table 1). Clinical examinations were negative for hepatomegaly, splenomegaly, K-F ring, and sunflower cataract. Other causes of serum aminotransferase elevation, such as viral hepatitis, muscle disorders, and hemolytic diseases were ruled out with proper investigations.

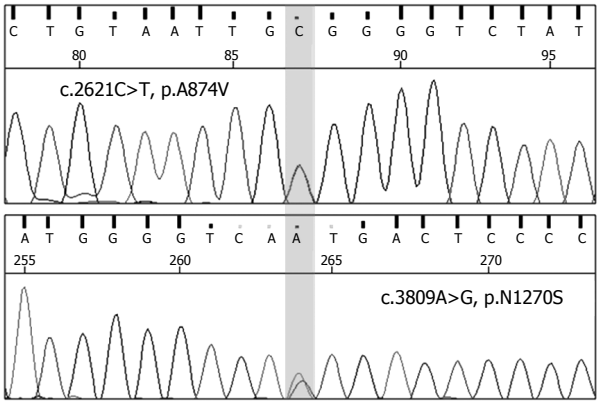

Figure 1 *ATP7B* gene sequencing detected two heterozygous mutations (p.A874V and p.N1270S) that have been reported to cause Wilson disease in the Wilson Disease Mutation Database (http://www.wilsondisease.med.ualberta.ca/database.asp), and predicted to be disease causing by Mutation Taster (http://www.mutationtaster.org).

The *ATP7B* gene sequencing detected two heterozygous mutations (p.A874V and p.N1270S) that have been reported to cause WD in the Wilson Disease Mutation Database (http://www.wilsondisease.med.ualberta.ca/database.asp), and predicted to be disease causing by MutationTaster (http://www.mutationtaster.org) (Figure 1). Sequencing also revealed 4 other heterozygous non-synonymous allels and 1 allel in the non-coding region, mutationtaster predicted them to be single nucleotide polymorphisms (SNPs). Both parents were screened for disease causing mutations and the SNP in the non-coding region. Father was heterozygous for the mutation of c.3809A>G (p.N1270S), and mother was heterozygous for c.2621C>T (p.A874V), and c.3903+6C>T (Table 2).

At the age of 23 mo, the patient was slightly undernourished with a weight of 11.5 kg (below 50th percentile by The WHO Child Growth Standards: http://www.who.int/childgrowth/standards/en/) and a height of 83 cm (below 15th percentile). At the last follow-up when the patient was 35 mo of age, liver function test was normal. Linear growth had significantly improved with weight for age reaching above the 50th percentile (14.5 kg), and height for age reaching above the 15th percentile (93 cm).

DISCUSSION

Shimizu *et al*[2] reported an 8-mo-old Japanese boy found to have low level of ceruloplasmin after mass screening. This child had normal liver function test results, and normal urinary copper excretion. However, the ATP7B gene sequencing detected a homozygous frame-shift mutation (c.2302insC). Kim *et al*[3] reported a 9-mo-old male infant with elevated aminotransferase detected after routine blood testing for acute diarrhea. The serum ceruloplasmin level was below normal range, but the 24-h urinary copper excretion was normal. Compound heterozygous

Table 1 Biochemical test results and treatment at various stages

Age (mo)	ALT (Normal range 0-40 IU/L)	AST (Normal range 0-40 IU/L)	GGT (Normal range 7-50 IU/L)	TBA (Normal range 0-10 umol/L)	Ceruloplasmin (Normal range 0.21-0.53 g/L)	Treatment
8	247	193	ND	ND	ND	
10	270	104	75	47.5	ND	
11	350	185	ND	ND	0.079	zinc 30 mg/d
14	152	83	ND	ND	ND	zinc 60 mg/d
24	43	30	22	2.9	ND	zinc 60 mg/d
35	37	26	ND	ND	ND	zinc 60 mg/d

ALP: Alkaline phosphatase; ALT: Alaninaminotransferase; AST: Aspartate amino transferase; GGT: Gamma glutamyl transpeptidase; TBA: Total bile acid; ND: Not done.

Table 2 *ATP7B* gene sequencing results

Exons	Heterozygous mutation/allel (parental origin)	SNP number	Status on WD mutation database	Mutation taster prediction (score)
Exon2	c.1216T>G, p.S406A (ND)	rs1801243	Non disease causing	Polymorphism (99)
Exon3	c.1366G>C, p.V456L (ND)	rs1801244	Not found	Polymorphism (32)
Exon10	c.2495A>G, p.K832R (ND)	rs1061472	Non disease causing	Polymorphism (26)
Exon11	c.2621C>T, p.A874V (Mother)	rs121907994/CM980173	Disease causing	disease causing (64)
Exon12	c.2855G>A, p.R952K (ND)	rs732774	Not found	Polymorphism (26)
Exon16	c.3419T>C, p.V1140A (ND)	rs1801249	Not found	Polymorphism (64)
Exon18	c.3809A>G, p.N1270S (Father)	rs121907990/CM994116, CM930060	Disease causing	disease causing (46)
Intron	c.3903+6C>T (Mother)	rs2282057	Not found	Polymorphism

Wilson Disease Mutation Database (http://www.wilsondisease.med.ualberta.ca/database.asp); Mutation Taster (http://www.mutationtaster.org); ND: Parental sequencing not done.

mutations were found with genetic analysis (known disease causing mutation of p.G1186S, and novel frameshift mutation of c.4006delA that resulted a stop codon).

The reason why this patient had such an early disease onset could be genetic, environmental, or combination of both. Two known disease causing mutations were detected in this patient, along with 5 other non-synonymous SNPs plus 1 SNP in the non-coding region. Non-synonymous SNPs might not be disease causing when appeared alone, but it is unknown whether it can contribute to disease process when appeared in combination with other disease causing mutations, or with other non-synonymous SNPs. Over consumption of copper could be considered as one of the causes of early WD onset. An epidemiologic study of serum copper levels in 8 provinces of China revealed that serum copper levels in people from eastern China were significantly higher than that of middle and western China[4]. Authors also conducted a survey proving that more frequent sea food consumption led to significantly higher copper levels in the body. Dietary and environmental factors may have played a role since coastal regions in eastern China are more industrialized, and people living there consumes more sea food than the people from other parts of the country. There is also evidence that long-term high copper intake in healthy men led to significantly higher copper retention in the body, and homeostatic regulation was not sufficient to maintain a normal copper absorption[5]. Our patient came from the coastal Shandong Province in eastern China, and potentially higher exposure to dietary and environmental copper, coupled with potentially severe disruption of copper homeostasis caused by *ATP7B* gene mutation plus SNPs may have led to enough copper accumulation, and caused liver damage at this young age. However, further studies need to be done in order to elucidate the complex interplay among genotype, phenotype, and environmental factors (such as diet and pollution).

In conclusion, an elevation of serum amino-transferase during infancy should always prompt pediatricians to exclude WD with genetic testing if other causes are negative.

COMMENTS

Case characteristics

An 8-mo-old Chinese boy presented with elevated liver enzymes.

Clinical diagnosis

Diagnosis of Wilson disease (WD) is made after a ceruloplasmin testing and *ATP7B* gene sequencing.

Differential diagnosis

Other causes of serum aminotransferase elevation, such as viral hepatitis, muscle disorders, and hemolytic diseases were ruled out with proper investigations.

Laboratory diagnosis

The serum ceruloplasmin level was extremely low, *ATP7B* gene sequencing revealed 2 disease causing mutations.

Imaging diagnosis
The liver and the gallbladder were normal on ultrasonography.

Treatment
Liver function improved and returned to normal after oral zinc gluconate therapy.

Related reports
The youngest WD child with normal liver function reported so far is an 8-mo-old Japanese boy, and the youngest child with elevated aminotransferase is a 9-mo-old Korean boy.

Experiences and lessons
An elevation of serum aminotransferase during infancy should always prompt pediatricians to exclude WD with genetic testing if other causes are negative.

Peer-review
Abuduxikuer *et al* have reported a case about an 8-mo-old Chinese boy that has been diagnosed with WD. The case report is interesting and factual.

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