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World Journal of Gastroenterology

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AIMS AND SCOPE

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (6): *Helicobacter pylori*

Helicobacter pylori and autoimmune disease: Cause or bystander

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Abstract

Helicobacter pylori (*H. pylori*) is the main cause of chronic gastritis and a major risk factor for gastric cancer. This pathogen has also been considered a potential trigger of gastric autoimmunity, and in particular of autoimmune gastritis. However, a considerable number of reports have attempted to link *H. pylori* infection with the development of extra-gastrointestinal autoimmune disorders, affecting organs not immediately relevant to the stomach. This review discusses the current evidence in support or against the role of *H. pylori* as a potential trigger of autoimmune rheumatic and skin diseases, as well as organ specific autoimmune diseases. We discuss epidemiological, serological, immunological and experimental evidence associating this pathogen with autoimmune diseases. Although over one hundred autoimmune diseases have been investigated in relation to *H. pylori*, we discuss a select number of papers with a larger literature base, and include Sjögrens syndrome, rheumatoid arthritis, systemic lupus erythematosus, vasculitides, autoimmune skin conditions, idiopathic thrombocytopenic purpura, autoimmune thyroid disease, multiple sclerosis, neuromyelitis optica and autoimmune liver diseases. Specific mention is given to those studies reporting an association of anti-*H. pylori* antibodies with the presence of autoimmune disease-specific clinical parameters, as well as those failing to find such associations. We also provide helpful hints for future research.

Key words: Autoimmunity; *Helicobacter pylori*; Infection; Gastritis; Mimicry; Rheumatology

Core tip: Multiple infectious agents have been implicated in the development of autoimmune disease. *Helicobacter pylori* is one pathogen which has been linked with multiple autoimmune diseases. This review will critically discuss a select few studies which have a larger evidence base, both in terms of positive and negative findings.

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INTRODUCTION

Autoimmune diseases arise from the interaction of genetic susceptibility and environmental exposures[1-4]. Among environmental exposures, infectious triggers have been implicated and studied extensively[1,5]. Infectious agents include bacteria, viruses and parasites, and may also consist of those organisms which comprise the normal flora[5]. Several mechanisms by which infectious agents may cause autoimmune disease have been proposed[6,7]. These include molecular mimicry[8-10], epitope spreading, bystander effect[11,12], microbial super-antigens, immune complex formation[13], MHC class Ⅱ expression on non-immune cells[14], direct inflammatory damage[13], high levels of pro-inflammatory cytokines such as interferon (IFN)-γ[10], and T-regulatory/Th17 imbalance.

Among infectious agents implicated, *Helicobacter pylori* (*H. pylori*) has received particular attention, in that it has been implicated in both organ specific and non-organ specific autoimmune disease[15]. As gastric disease in relation to *H. pylori* has been discussed extensively in multiple reviews and studies[16-18], it will not be discussed in this review. Likewise, multiple other autoimmune conditions have been linked with *H. pylori*, with evidence bases of varying content. In fact, amongst the autoimmune or autoimmune related diseases listed by AARDA (American Autoimmune Related Diseases Association, http://www.aarda.org/), 95 have been studied sporadically or systematically in regard to their connection with *H. pylori*, while among the remaining 61 there are no studies (yet) in Pubmed (search up to 29 September 2013) (Tables 1 and 2). Therefore, this review will discuss selected autoimmune conditions, both organ specific and non-organ specific, which have an evidence base (positive or negative) in relation to *H. pylori* infection. Amongst the non-organ specific autoimmune disorders, we thoroughly discuss immune thrombocytopenic purpura (ITP) and autoimmune rheumatic diseases, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), Sjögren syndrome (SjS), systemic sclerosis (SSc). Amongst the organ specific diseases linked with *H. pylori*, autoimmune thyroid disease (AiTD), and multiple sclerosis (MS)/neuromyelitis optica (NMO) are discussed, as well as autoimmune liver diseases such as primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC) and autoimmune hepatitis (AIH). Although a wealth of literature is available for some conditions, we present selected papers that highlight the current findings, or lack thereof. It will become apparent that the evidence in support of *H. pylori* as a cause of some autoimmune conditions varies from one condition to the next.

POTENTIAL MECHANISMS OF *H. PYLORI*-INDUCED AUTOIMMUNITY

Several mechanisms of pathogen-induced autoimmunity have been described in studies of *H. pylori*-induced autoimmunity[19]. We briefly discuss some of these papers, starting with the study by Jackson and colleagues[20]. These investigators found that chronic *H. pylori* infection was associated with an increased risk of an elevated serum C-reactive protein, indicating an ongoing inflammatory state. This chronic inflammation may result in ongoing antigenic stimulation, and induces a systemic inflammatory response, and therefore extra-gastrointestinal disease[20]. However, such hypotheses are not accompanied by solid experimental data. We need to emphasize that this, as well as most other studies investigating the role of *H. pylori,* speculates rather than demonstrates a pathogenic role for this bacterium. Another study found that molecular mimicry of *H. pylori* antigens activated cross-reactive T cells in autoimmune gastritis[21]. *H. pylori* components (especially urease) have been shown to activate B cells to produce IgM rheumatoid factor, anti-ds-DNA, and anti-phospholipid choline antibodies[22]. The former studies belong to those few (compared to the great majority of the studies) that to some extent provide a mechanistic approach as to how the pathogen can inflict loss of immunological tolerance, which is an important component for the initiation of antigen-driven autoimmunity. Similar mechanisms have been proposed in relation to heat shock protein (hsp) 60[23]. Another piece of evidence which can support the major role of *H. pylori* in the development of autoimmune diseases (and not just in the induction of autoantibodies) stems from studies on animal models of autoimmune diseases. Infection of male C57BL/6 mice with *H. pylori* can induce a disease that resembles human PBC[24]. However, most animal models of autoimmune diseases do not rely on *H. pylori* infection for the induction of the disease or do not provide data to support that this pathogen is needed for disease development. Most of the mechanisms discussed in the literature remain as hypotheses that require more extensive investigation.

H. PYLORI AND AUTOIMMUNE RHEUMATIC DISORDERS

The pathogenetic evidence linking *H. pylori* with autoimmune rheumatic diseases varies amongst diseases. For example, while there are a reasonable number of studies investigating this topic in SjS, the data stemming from SLE are relatively few and inconsistent. There are several explanations that could account for the great variation in the number of the studies conducted amongst diseases. Some studies are rare and translational research is difficult to perform, as in for example the case of SSc. Other diseases do not have reliable animal models, and in these dis-

Table 1 Autoimmune diseases or autoimmune disease-related disorders which have been studied for their possible (direct or indirect) relation with *Helicobacter pylori* infection

	AID or AID-related disorders linked to *H.pylori*		AID or AID-related disorders linked to *H.pylori*
1	Alopecia areata	49	Juvenile diabetes (Type 1 diabetes)
2	Antiphospholipid syndrome	50	Kawasaki syndrome
3	Autoimmune angioedema	51	Leukocytoclastic vasculitis
4	Autoimmune hepatitis	52	Lichen planus
5	Autoimmune hyperlipidemia	53	Linear IgA disease
6	Autoimmune hemolytic anemia	54	Lupus (SLE)
7	Autoimmune myocarditis	55	Microscopic polyangiitis
8	Autoimmune oophoritis	56	Mixed connective tissue disease
9	Autoimmune pancreatitis	57	Mooren's ulcer
10	Autoimmune polyglandular syndromes	58	Multiple sclerosis
11	Autoimmune thrombocytopenic purpura	59	Myositis
12	Autoimmune thyroid disease	60	Narcolepsy
13	Autoimmune urticaria	61	Neuromyelitis optica (Devic's)
14	Axonal and neuronal neuropathies	62	Neutropenia
15	Behcet's disease	63	Ocular cicatricial pemphigoid
16	Bullous pemphigoid	64	Optic neuritis
17	Cardiomyopathy	65	Palindromic rheumatism
18	Celiac disease	66	Pars planitis (peripheral uveitis)
19	Chagas disease	67	Pemphigus
20	Chronic inflammatory demyelinating polyneuropathy	68	Peripheral neuropathy
21	Chronic recurrent multifocal osteomyelitis	69	Perivenous encephalomyelitis
22	Crohn's disease	70	Pernicious anemia
23	Cogans syndrome	71	Polyarteritis nodosa
24	Demyelinating neuropathies	72	Polymyalgia rheumatica
25	Dermatitis herpetiformis	73	Polymyositis
26	Dermatomyositis	74	Primary biliary cirrhosis
27	Devic's disease (neuromyelitis optica)	75	Primary sclerosing cholangitis
28	Eosinophilic esophagitis	76	Psoriasis
29	Eosinophilic fasciitis	77	(Idiopathic) pulmonary fibrosis
30	Erythema nodosum	78	Pyoderma gangrenosum
31	Experimental allergic encephalomyelitis	79	Raynaud's phenomenon
32	Fibromyalgia	80	Reactive Arthritis
33	Fibrosing alveolitis	81	Reiter's syndrome
34	Giant cell arteritis (temporal arteritis)	82	Relapsing polychondritis
35	Giant cell myocarditis	83	Rheumatoid arthritis
36	Glomerulonephritis	84	Sarcoidosis
37	Goodpasture's syndrome	85	Scleroderma (systemic sclerosis)
38	Graves' disease	86	Sjogren's syndrome
39	Guillain-Barre syndrome	87	Temporal arteritis/Giant cell arteritis
40	Hashimoto's thyroiditis	88	Thrombocytopenic purpura
41	Henoch-Schonlein purpura	89	Transverse myelitis
42	Hypogammaglobulinemia idiopathic thrombocytopenic purpura	90	Type 1 diabetes
43	IgA nephropathy	91	Ulcerative colitis
44	IgG4-related sclerosing disease	92	Undifferentiated connective tissue disease
45	Immunoregulatory lipoproteins	93	Uveitis
46	Inclusion body myositis	94	Vasculitis (other forms)
47	Interstitial cystitis	95	Vesiculobullous dermatosis
48	Juvenile arthritis		

The list includes diseases in alphabetic order as they have been deposited in the official website of AARDA (American Autoimmune Related Diseases Association) with minor revisions. Diseases with at least one study (Pubmed Search) investigating *Helicobacter pylori* (*H. pylori*) as a trigger have been included. AID: Autoimmune disease.

orders it has been almost impossible to assess the role of infectious agents in the induction of autoimmunity. Also, for some diseases the prevailing idea amongst researchers has been that *H. pylori* is not an attractive etiologic agent, and this has prevented more research in this topic over the years. Nevertheless, epidemiological, serological and clinical studies have been performed to some extent and are reviewed herein.

Sjögren's syndrome

SjS is an autoimmune condition characterized by lymphoid cell infiltration and destruction of exocrine glands[19]. As lacrimal and salivary glands are most affected, a link with *H. pylori* has been made given its prevalence in the oral cavity[19], which may be associated with anti-*H. pylori* antibodies[25].

Aragon *et al*[23] found that 79.4% of SjS patients had anti-*H. pylori* antibodies, and that 88.2% had anti-hsp60. This was significantly higher than other autoimmune controls (18.2% with anti-*H. pylori*; 27.3% with anti-hsp60), and healthy controls (48.8% anti-*H. pylori*; 37.2% anti-hsp60)[23]. El Miedany *et al*[26] failed to find statistically significant differences in the prevalence of anti-*H. pylori*

Table 2 Autoimmune diseases or autoimmune diseases-related disorders which have not been studied for their possible (direct or indirect) relation with *Helicobacter pylori* infection

	AID or AID-related disorders not linked to *H. pylori*
1	Acute Disseminated Encephalomyelitis
2	Acute necrotizing hemorrhagic leukoencephalitis
3	Addison's disease
4	Agammaglobulinemia
5	Amyloidosis
6	Ankylosing spondylitis
7	Anti-GBM/Anti-TBM nephritis
8	Autoimmune aplastic anemia
9	Autoimmune dysautonomia
10	Autoimmune immunodeficiency
11	Autoimmune inner ear disease
12	Autoimmune retinopathy
13	Balo disease
14	Castleman disease
15	Chronic fatigue syndrome
16	Churg-Strauss syndrome
17	Cicatricial pemphigoid/benign mucosal pemphigoid
18	Congenital heart block
19	Coxsackie myocarditis
20	CREST disease
21	Essential mixed cryoglobulinemia
22	Discoid lupus
23	Dressler's syndrome
24	Endometriosis
25	Evans syndrome
26	Granulomatosis with Polyangiitis (formerly called Wegener's Granulomatosis)
27	Hashimoto's encephalitis
28	Herpes gestationis
29	Juvenile myositis
30	Lambert-Eaton syndrome
31	Lichen sclerosus
32	Ligneous conjunctivitis
33	Lyme disease,
34	(Chronic) Meniere's disease
35	Mucha-Habermann disease
36	Myasthenia gravis
37	Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcus
38	Paraneoplastic cerebellar degeneration
39	Paroxysmal nocturnal hemoglobinuria
40	Parry Romberg syndrome
41	Parsonage-Turner syndrome
42	POEMS syndrome
43	Postmyocardial infarction syndrome
44	Postpericardiotomy syndrome
45	Progesterone dermatitis
46	Psoriatic arthritis
47	Pure red cell aplasia
48	Reflex sympathetic dystrophy
49	Restless legs syndrome
50	Retroperitoneal fibrosis
51	Rheumatic fever
52	Schmidt syndrome
53	Scleritis
54	Sperm and testicular autoimmunity
55	Stiff person syndrome
56	Subacute bacterial endocarditis
57	Susac's syndrome
58	Sympathetic ophthalmia
59	Takayasu's arteritis
60	Tolosa-Hunt syndrome
61	Vitiligo

The list includes diseases in alphabetical order as they have been deposited in the official website of AARDA (American Autoimmune Related Diseases Association) with minor revisions. Diseases with at least one study (Pubmed Search) investigating *Helicobacter pylori* (*H. pylori*) as a trigger have been included. AID: Autoimmune disease.

antibodies between patients with primary and secondary SjS (80.6% *vs* 71% for IgG, and 47.2% *vs* 38.7% for IgA, respectively). However, anti-*H. pylori* antibodies were significantly less prevalent in patients with connective tissue disorders lacking sicca syndrome symptomatology (60.9% for IgG and 19.6% for IgM). The lowest prevalence of IgG and IgM anti-*H. pylori* antibodies was found in normal controls (56.3% for IgG and 12.5% for IgM, respectively)[26]. Similar results have been found in further studies[27], but contradictory data have been provided in others[28]. A study by the group of Theander[28] examined the prevalence of *H. pylori* in a Swedish cohort of 164 SjS patients, and found that 45% were seropositive for *H. pylori* infection, including 23% with anti-CagA antibodies. However, these rates were lower than those seen in a control group of orthopedic outpatients without autoimmune conditions, and similar to rates found among healthy individuals[28]. That group therefore concluded that *H. pylori* infection was not linked with SjS[28].

Some studies have attempted to link evidence of *H. pylori* infection with clinical features of SjS. For example, El Miedany *et al*[26] have found that there is a significant correlation between (IgG and IgM) anti-*H. pylori* antibody seropositivity and the presence of primary and secondary SjS, as well as various clinical parameters. Logistic regression analysis has revealed that the presence of IgG anti-*H. pylori* antibodies significantly correlates with age, disease duration and global score for disease status.

Another possible link between SjS and *H. pylori* may be found in mucosa-associated lymphoid tissue (MALT) lymphomas that may arise from chronic antigenic stimulation (*i.e.*, chronic infection and/or autoimmune disease). *H. pylori* was detected in gastric tissue from MALT, and interestingly, there is an increased incidence of MALT lymphomas and marginal zone B cell neoplasms in SjS[29]. It is possible that *H. pylori* eradication in SjS may result in decreased incidence of MALT, as is the case for gastric MALT lymphomas[30-32]. Further studies regarding the prevalence of *H. pylori* in SjS in different populations are currently needed, in addition to monitoring for *H. pylori* in at-risk individuals.

Rheumatoid arthritis

Sir James Paget was one of the very first to consider the possibility that what is now known as rheumatoid arthritis may indeed be caused by microbial infections. In 1853, Paget hypothesized that all diseases that manifest their symptoms symmetrically, such as "the deformities of chronic rheumatism", must be blood-borne and could be caused by a demonstrable virus. *H. pylori* has been considered one of the infectious agents linked to RA; however, the data do not support this. An increased incidence of peptic ulcer disease in RA patients is most likely related to the use of non-steroidal anti-inflammatory drugs[33]. Yamanishi *et al*[22] found increased IgM rheumatoid factor in B cells chronically stimulated with *H. pylori* urease. However, several studies demonstrated that there is a lower prevalence of *H. pylori* in RA patients, and other studies found the prevalence of *H. pylori* to be similar to that of the healthy controls[27,34,35]. After *H. pylori* eradication, no change in RA symptoms was reported by several studies[36-38], although improvement was noted in others[39,40]. Currently, the data are mixed regarding RA and *H. pylori*, and it appears that the link is weak.

Systemic lupus erythematosus

H. pylori prevalence has been studied in patients with SLE, but the results vary amongst reports. A recent study has failed to find significantly higher prevalence of anti-*H. pylori* antibodies in SLE patients compared to controls[41]. Of note, this study showed an increased prevalence of anti-*H. pylori* antibodies in patients with anti-phospholipid syndrome, giant cell arteritis, SSc and PBC[41]. Such findings have also been reported in the past. Kalabay *et al*[42] have studied the prevalence of anti-*H. pylori* antibodies in various autoimmune rheumatic diseases. These authors have found comparable prevalence of this pathogen in patients with SLE and healthy controls (57% *vs* 59%)[42]. The highest prevalence of anti-*H. pylori* antibodies was found in patients with undifferentiated connective tissue disorders (82%)[42]. Of interest, an early study reported a negative association between *H. pylori* seropositivity and the development of SLE in African-American women[43]. In particular, female African-American patients with SLE had a lower prevalence of *H. pylori* seropositivity compared to controls (38.1% *vs* 60.2%). That study also found that seronegative African-American females were more likely to develop SLE, and at an earlier age than their seropositive counterparts[43]. Thus, the mean age of onset for SLE was 34.4 years in the seropositive group and 28 years in the seronegative group. These data suggest that either the presence of the pathogen confers protection from SLE or that the same mechanisms that make individuals prone to *H. pylori* infection also promote the immune dysregulation which is necessary for SLE's induction in African-American females.

Much like RA, the role of *H. pylori* in SLE is also inconclusive. In an animal model, urease exposure induced anti-ssDNA antibody production[22]. However, low anti-*H. pylori* antibodies have been found in SLE patients, with levels comparable to healthy controls[27,43]. Overall, the evidence does not support a role for *H. pylori* in the development of SLE[44].

Systemic sclerosis

Dysregulation of innate and adaptive (humoral and cellular) immunity plays an important role in the induction of SSc[45-47]. The very low concordance rate for SSc in monozygotic twins has led investigators to consider that the pathogenesis of this disease rests more in the effect of environmental factors (including viruses and bacteria) rather than genetic influences[48].

In a Japanese cohort of SSc patients, IgG antibodies against *H. pylori* were found in 55.6% of the patients, a

prevalence significantly higher compared to that in the control group[49]. Another Japanese study found a similar prevalence of these antibodies (57.8%), and also a higher prevalence of reflux esophagitis amongst anti-*H. pylori* antibody-positive patients compared to anti-*H. pylori* antibody-negative patients[50]. Others have also noted an increased rate of *H. pylori* infection in patients with SSc compared to controls[15,23,51,52]. However, a significant number of studies has failed to find an increased prevalence of *H. pylori* seropositivity compared to control groups, further indicating the lack of conclusive data regarding the extent by which *H. pylori* confers susceptibility to SSc[53-56].

Of clinical relevance, early data have indicated that *H. pylori* eradication improves Raynaud's phenomenon in patients with SSc[57,58]. Another study has noted that skin involvement appears to be a predominant feature of *H. pylori*-infected SSc patients compared to their seronegative counterparts. No other clinical parameters, including the distribution of sex, age, disease duration, autoantibody profile, estimated pulmonary artery systolic pressure, hemoglobin, ESR, renal and liver function indices were different between *H. pylori*-infected or non-infected SSc patients[59]. On the other hand, SSc patients with Barrett's esophagus appear less likely to be *H. pylori*-positive compared to SSc patients without Barrett's esophagus (10% *vs* 42.5%). Such findings have underlined the potential protective role of *H. pylori* for the development of Barrett's esophagus[60]. In pathophysiological terms, the results of the data discussed so far could be interpreted as follows: (1) *H. pylori*-infected patients are more prone to develop SSc; (2) SSc patients are more susceptible to infection by *H. pylori*, probably due to the disturbed gastrointestinal motility which is a characteristic feature of SSc; and (3) after the development of SSc (probably caused by reasons other than H pylori), infection with the pathogen protects the affected patients from unwanted complications (such as Barrett's esophagus).

Danese *et al*[56] have tackled the topic from another corner. While they failed to find a difference in the prevalence of the pathogen between SSc patients and controls, they reported that 90% of the *H. pylori*-positive SSc patients were infected with the virulent CagA strain compared to just 37% of the non-CagA seropositive controls. Elevated levels of anti-hsp65 (but not of anti-hsp60) *H. pylori* antibodies have been found in SSc patients compared to controls[42].

Vasculitides

Data on the potential link between *H. pylori* and vasculitides are very limited. For example, we know very little about the role of this pathogen in granulomatosis with polyangiitis (GPA), formerly known as Wegener's granulomatosis. A serological study has shown that anti-*H. pylori* antibodies are more prevalent in GPA compared to controls[61]. Such findings may be of biological significance as *H. pylori* has been considered a potential trigger of vascular inflammation. Thus, the SS1 strain of *H. pylori*-infected heterozygous low density-lipoprotein receptor (LDLR)+/- apoE apolipoprotein E (apoE)+/- mice develop autoimmune inflammation, platelet activation and atherosclerosis[62]. A role for the pathogen in atherosclerosis and vasculitis has been suggested but there is no general agreement on this issue[63]. A previous report was unable to identify significant differences in the rate of anti-*H. pylori* antibodies between patients with GPA and control diseases[64]. The study by Lidar *et al*[61] failed to find any association between anti-*H. pylori* antibody seropositivity in healthy controls and polyarteritis nodosa, microscopic polyangiitis, eosinophilic granulomatosis with polyangiitis (EGPA), also known as Churg Strauss syndrome, and giant cell arteritis[61].

Another study reported disappearance of antiphospholipid syndrome after *H. pylori* eradication[65], but data are too limited on the issue to draw any conclusions.

IMMUNE-MEDIATED SKIN DISORDERS

H. pylori infection has been considered a potential inducer of several immune-mediated skin disorders. These disorders can be manifestations of systemic vasculitides (Behçet's disease) or may be related to skin disorders with presumed autoimmune origin (psoriasis, alopecia areata, lichen planus, *etc.*). Due to space constraints, this review will discuss the role of *H. pylori* in selected skin disorders including psoriasis, alopecia areata and Behçet's disease. Other skin disorders linked to *H. pylori* include, amongst others, atopic dermatitis, chronic or nodular prurigo, recurrent aphthous stomatitis, rosacea, chronic urticaria, lichen planus, and Sweet's syndrome, and are reviewed elsewhere[66]. We will also discuss the link between *H. pylori* and chronic urticaria, as a plethora of data have been obtained and the outcomes of these studies are extremely helpful for the understanding of the interactions between the pathogen and the host.

Psoriasis

Psoriasis affects 1%-3% of Caucasians. The etiology of the disease remains poorly understood, although immune-mediated mechanisms appear to play a significant role in the development of the disease, including exposure to particular pathogens.

To this end, several studies have investigated a possible link between *H. pylori* and psoriasis[67-74].

Anti-*H. pylori* antibodies have been reported to be more prevalent in psoriatic patients compared to controls. For example, Qayoom *et al*[72] have reported that 40% of psoriatic patients and only 10% of healthy controls (all without known upper gastrointestinal symptoms) had anti-*H. pylori* antibodies. However, other studies have failed to find any difference in the prevalence of *H. pylori*[70].

A large study from Turkey, investigating 300 psoriatic patients and 150 controls, has reported comparable prevalence of *H. pylori* infection in patients and controls.

However, the same study suggested that *H. pylori* status relates to clinical parameters[75], as it was able to show that patients lacking *H. pylori* had less severe psoriatic disease compared to the seropositive cases. Also, all patients with moderate or severe psoriasis were *H. pylori*-positive. Intriguingly, patients treated for both psoriasis (with acitretin) and for *H. pylori* (eradication therapy) showed more rapid improvement of the skin disease, compared to those treated with acitretin only. Notably, psoriasis was also improved in patients receiving only eradication treatment[75]. This study confirmed anecdotal reports or case studies showing that eradication therapy improves psoriasis[73,76].

Strains of *H. pylori* that express the cytotoxin-associated gene A (CagA) have been associated with a more virulent disease and are believed to play an important role in the clinical outcome of the infection. Several authors have considered that links between the pathogen and autoimmunity may differ in accordance to the virulence of the infecting strain. This has also been the case for *H. pylori* and psoriasis. To this end, Daudén *et al*[68] were unable to find any difference in terms of CagA seropositivity between psoriatic patients and patients with non-ulcer dysplasia (54.5% *vs* 68.1%, respectively).

Chronic urticaria

The pathogenic role of *H. pylori* infection has been extensively studied in chronic urticaria. Though this disease cannot be considered a typical autoimmune disease, it is of interest to discuss the findings provided so far, as these may help us understand the role of this pathogen in the development of immune-mediated pathologies. Investigations have not been limited to the prevalence of infection[66], but have been extended to include the role of eradication therapy in the clinical course of chronic urticaria[77-86]. Selected papers give us an insight into the extent by which the pathogen and its eradication influence the clinical outcome of the disease. For example, recurrence of urticaria following re-infection by *H. pylori* has been reported[87]. On the other hand, chronic urticaria has also been described upon administration of eradication therapy for *H. pylori* infection[79]. Nevertheless, some patients with chronic spontaneous urticaria are resistant to conventional doses of antihistamine medications. A subgroup of those (approximately 28%) receiving both eradication therapy and antihistamines show significant decrease of the Urticaria Activity Score and complete loss of their urticaria symptoms, suggesting that treatment for *H. pylori* makes these patients less resistant to antihistamines[77]. These findings are in agreement with other studies reporting an overall improvement of chronic urticaria following administration of eradication therapy for *H. pylori*[88-90]. Other studies have failed to find any relationship between eradication therapy and clinical phenotypes[91]. Of interest, a recent comprehensive review utilized the Grading of Recommendations Assessment, Development, and Evaluation approach to analyze and determine the quality of evidence for this proposed therapy. Their analysis has included 10 trials showing a benefit and 9 trials failing to report a benefit of *H. pylori* eradication therapy. This analysis reached the conclusion that the evidence provided so far that *H. pylori* eradication leads to improvement of chronic urticaria outcomes is weak and conflicting. Negative studies showing no benefit in the course of chronic urticaria also led to an overall very low grade of confidence. *H. pylori* virulent genotypes in the urticaria patients do not appear to affect the clinical course of the disease[92].

Behçet's disease

The role of *H. pylori* infection in Behçet's disease (BeD) remains controversial[93-95]. Most studies originate from Turkey, a country with a high incidence of BeD. Avci *et al*[95] have failed to find an association between *H. pylori* and BeD. Other studies published in the form of abstracts or in Turkish journals have published inconsistent results reporting comparable or higher prevalence rates of *H. pylori* infection in patients with BeD[93]. One study also from Turkey reported an increased seropositivity of *H. pylori* cytotoxin-associated gene-A in patients with BeD[96].

Improvement of BeD features in patients receiving eradication therapy has also been reported[95], and includes improvements in the cutaneous lesions, arthritis/arthralgia and oral or genital ulcers. The limited number of studies prevents safe conclusions as to the potential links.

Alopecia areata

AA is an immune-mediated disorder characterized by hair loss. The disease affects all ethnic groups, ages, and both sexes. Attempts to investigate the role of *H. pylori* in this disease have been very few and led to inconclusive results[97,98]. Seroprevalence rates of *H. pylori* infection in patients with AA are increased or not compared to controls[97,99]. Eradication of *H. pylori* in AA has also been proposed[100], but not studied extensively.

IMMUNE THROMBOCYTOPENIC PURPURA

ITP may occur by itself (idiopathically) or secondary to another condition, including autoimmune conditions (namely AiTD, SLE, anti-phospholipid syndrome). Although the prevalence of *H. pylori* in ITP patients has been found to be similar to controls[101], improvements in platelet counts following *H. pylori* eradication have been reported[102-107]. Suzuki *et al*[106] reported that the platelet response was more pronounced in those patients with the CagA-positive *H. pylori* strain. Interestingly, anti-CagA antibodies cross-react with peptides expressed on platelets of ITP patients[108]. These findings have led to the suggestion of eradication of *H. pylori* for the treatment of ITP[109]. Takahashi *et al*[110] reported that platelet-associated IgG declined after *H. pylori* eradication, as did

molecular mimicry with the CagA region. In that study, *H. pylori* was found in 75% (15 of 20 patients) of ITP patients of Japanese descent, and eradicated in 87% (13 of 15)[110]. Increased platelet count was observed in 54% (7 of 13) of patients within four months of eradication[110]. Over a dozen other studies have also indicated an improvement in platelet count following *H. pylori* eradication, and are well-reviewed by Hernando-Harder and colleagues[66]. Platelet eluates from 12 ITP patients recognized *H. pylori* CagA, although it should be noted that three of the 12 patients were seronegative for *H. pylori* infection[110]. Levels of anti-CagA antibodies declined in three patients following *H. pylori* eradication. This latter result suggested a role for cross-reactivity and molecular mimicry[110].

The role of molecular mimicry and cross reactivity between *H. pylori* components and self-peptides is not new, as antibodies against the H/K-ATPase in the gastric mucosa have been found to be generated *via* molecular mimicry with *H. pylori* in atrophic gastritis[111]. Molecular mimicry has been considered a mechanism that could explain other *H. pylori*-induced autoimmune phenomena, but very few studies have addressed this in an experimental way. The role of CagA strains is also under investigation in other conditions[112,113].

AUTOIMMUNE THYROID DISEASE

A larger amount of data links *H. pylori* infection with AiTD, and in particular with Graves' disease[114]. Bassi and colleagues[115] aimed to correlate the CagA strain of *H. pylori* with AiTD by investigating 112 consecutive patients at first diagnosis of AiTD. Those researchers tested for *H. pylori* in stool samples (to confirm ongoing infection), and CagA in serum samples. *H. pylori* and Graves' disease were associated (83.7% patients were *H. pylori* seropositive). No association was found with Hashimoto's thyroiditis[115]. Most patients (89.2%) seropositive for *H. pylori* were infected with the CagA strain[115]. This was in accordance with a previous study by the same group[116]. Negative findings in regard to Hashimoto's were reported in other studies[103,117], while some reported a positive association[114,118,119].

Cross-reactivity between bacterial and thyroid antigens has been proposed as a mechanism in *H. pylori*-induced AiTD[120]. Indeed, amino acid sequence similarities between CagA *H. pylori* and thyroid peroxidase have been reported[121], and one group described a reduction in thyroid autoantibodies following *H. pylori* eradication[122]. Larizza *et al*[123] suggests that *H. pylori* may induce or worsen Graves' disease in patients carrying HLA-DRB10301, and further suggested eradication in certain risk groups. These findings do suggest a possible causative link between the CagA strain of *H. pylori* and the development of Graves' disease, but deserve further research. It should be noted that AiTDs are often found concomitantly with other autoimmune conditions, and that the link between the pathogen and autoimmune thyroiditis may indeed reflect a potential contribution of *H. pylori* in the simultaneous induction of multiple autoimmune diseases in susceptible individuals[124]. The exact mechanisms by which exposure to a microbe elicit more than one autoimmune manifestations are not well defined but cross-reactive responses against a microbial mimic and several self-antigens have been documented[125-127], and may account for this. The reverse is also possible, whereby an autoepitope is cross-reactively targeted by several unrelated microbial mimics in a "multiple hit" scenario[128,129].

MULTIPLE SCLEROSIS AND NEUROMYELITIS OPTICA

H. pylori infection has been considered the likely trigger of various neurological disorders of the central nervous system including MS/NMO, Alzheimer's disease, Parkinson's disease, seizure disorders, cerebrovascular diseases, mild cognitive impairment, migraine and ophthalmic disorders, as reviewed elsewhere[130]. A large amount of data has been reported regarding *H. pylori* and MS/NMO. A recent study by Long *et al*[131] determined *H. pylori* infection status in a cohort of 2 NMO patients, 17 at high risk of NMO, 42 MS and 27 healthy controls. *H. pylori* antibodies were found in 90.4% NMO, 95.8% high-risk NMO, 73.8% MS, and 59.3% controls[131]. There was no statistically significant difference between the MS and control group ($P = 0.726$)[131]. Interestingly, 93% of patients with aquaporin-4 antibodies were also seropositive for *H. pylori*[131]. Yoshimura *et al*[132] analyzed 116 NMO patients for various antibodies to infectious agents, as well as for seropositivity for aquaporin-4 antibodies. They found that *H. pylori* infection was associated with anti-aquaporin-4 antibody positivity[132]. Similar findings were also reported in other studies[133-135].

Several studies found a lower prevalence of *H. pylori* amongst MS patients compared to controls. Mohebi and colleagues noted a lower prevalence of *H. pylori* in a cohort of MS patients[136], in a study which analyzed 163 MS patients and 150 controls for anti-*H. pylori* IgG and IgM. Seropositive *H. pylori* patients had a lower MS incidence and fewer neurological complications[136]. Wender also noted a lower anti-*H. pylori* prevalence in MS *vs* controls[137]. Li *et al*[138] evaluated 105 MS patients and 85 controls for antibodies against *H. pylori* in sera. The MS group was sub-divided into 52 opticospinal MS and 53 conventional MS. In the conventional MS group, 22.6% of patients were positive for anti-*H. pylori*, compared to 51.9% of opticospinal MS and 42.4% of controls[138]. These data suggest a potential link between NMO and *H. pylori*, although this does not appear to be the case in MS.

AUTOIMMUNE LIVER DISEASES

Some *Helicobacter* species, including *H. hepaticus*, *H. pul-*

lorum and *H. billis*, are more bile-tolerant compared to *H. pylori*, and can survive in very low concentrations in human bile[139]. This finding has prompted investigators to consider that *Helicobacter* species other than *H. pylori* are potential inducers of hepatocyte and biliary epithelia cell autoimmunity. Nevertheless, studies have addressed the role of *H. pylori* in autoimmune liver diseases, and provided interesting data.

The role of *H. pylori* has been studied mainly in PBC, an autoimmune cholestatic liver disease characterized by the immune-mediated destruction of small intrahepatic bile ducts. Some studies have also been conducted in PSC, another autoimmune cholestatic disease affecting the larger bile ducts. Studies on the role of this pathogen in the induction of AIH, an autoimmune liver disease affecting hepatocytes, are very limited.

Primary Biliary Cirrhosis

Tanaka *et al*[140] have failed to detect *H. pylori* in liver tissues from patients with PBC. Others have been able to detect *H. pylori* in PBC livers, although this was in a minority of samples tested[140].

Researchers have assessed the seroprevalence of *H. pylori* in PBC and identified significant differences amongst patients and controls[15]. For example, Shapira *et al*[41] reported anti-*H. pylori* antibodies in 54% of patients with PBC compared to 31% ($P < 0.01$) of patients with other conditions, while Tanaka *et al*[140] have failed to find any differences between patients and demographically-matched controls (51% *vs* 46%, respectively).

Our group has assessed the role of molecular mimicry between *H. pylori* and PBC-specific autoantigens and identified through database searches a significant amino acid sequence similarity between the major mitochondrial autoepitopic region from pyruvate dehydrogenase complex E2 subunit and urease beta of *H. pylori*[141]. However, we have failed to find any evidence of immunological cross-reactivity at the B-cell level[141]. We also tested the identified mimics as targets of CD4 T-cell responses, and we did not find any significant T-cell recognition[142]. In a subsequent study, we investigated the potential role of cross-reactive antibodies against *H. pylori* VacA antigen and human PDC-E2, but the results were also negative, clearly demonstrating that these two *H. pylori* antigens are unlikely candidates as cross-reactive targets in molecular mimicry mechanisms involved in PBC[143].

Primary Sclerosing Cholangitis

An early study in Scandinavian PSC patients indicated detectable *H. pylori* DNA in livers from patients with PSC and other liver diseases[140]. This has promoted a series of subsequent studies investigating the role of *Helicobacter* species in PSC and other autoimmune liver diseases. Krasinskas *et al*[144] detected *Helicobacter* DNA in 9 of 56 (16%) PSC patients by 16SrRNA PCR, including 7 (12.5% of the total), in whom there was evidence of *H. pylori* CagA by PCR. Recent PCR analyses have indicated that *H. pylori* or other *Helicobacter* species can be detected in up to 13% of liver tissue specimens from pediatric patients with autoimmune sclerosing cholangitis (an autoimmune form of PSC firstly noted in children) and AIH[145]. The same authors detected in the past *H. pylori* (but not other *Helicobacter* species) in liver tissues from PBC and adult PSC patients[140].

As PSC patients frequently suffer from ulcerative colitis, it has been hypothesized that alteration in the gut flora due to UC-related intestinal inflammation may promote gut translocation of *Helicobacter* to the liver. Gut translocation of pathogens appears an attractive mechanism for the induction of liver autoimmunity and there are some data in support of its validity[146,147].

The prevalence of anti-*H. pylori* antibodies does not differ between pediatric PSC patients (6.6%) and controls (4%-10% depending on the age)[145]. In fact, an increased prevalence of antibodies against non-gastric anti-*H. pylori* antibodies has been noted in patients with autoimmune liver diseases[148].

Autoimmune Hepatitis

The prevalence of anti-*H. pylori* antibodies does not appear to differ between patients with AIH (pediatric or adult) and controls[149-151]. Also, *H. pylori* DNA can be found in a minority of liver tissue samples from patients with AIH with no difference between patients and controls. Currently, there is insufficient evidence to link *H. pylori* with AIH.

UNMET CHALLENGES AND EXPERIMENTAL DOWNSIDES

The role of infectious agents in the development of autoimmune disease has been studied extensively. *H. pylori* is included among those organisms that have been investigated, although findings vary from one condition to the next. Large amounts of data suggest a plausible link with AiTD, NMO, ITP and psoriasis. Less evidence is present regarding RA, SLE, BeD, PBC, AIH and MS. There is inconclusive evidence regarding SjS, SSc, PSC and AA. Table 3 gives an overview of the major findings in support or against the implication of *H. pylori* in the development of these diseases.

Idiopathic diseases with an autoimmune component have been the focus of investigation in regard to the role of *H. pylori*. For example, an autoimmune form of idiopathic dysrhythmias has been linked specifically with CagA and VacA-positive *H. pylori* strains[152]. This indicated the potential of the pathogen to be linked with conditions now considered "idiopathic". Also, parasitic diseases such as the *Trypanosma cruzi*-induced Chagas disease need to be revisited, especially under recent developments showing not only that a proportion of these patients present with autoimmune features but also because such patients are also co-infected with *H. pylori* strains[153]. In addition, other conditions that are now considered to

Table 3 Evidence in support or against the role of *Helicobacter pylori* in autoimmune disease

Autoimmune condition	Evidence in support and/or against the role of *H. pylori*	Overall opinion
SjS	Support: Oral cavity populated with *H. pylori* Higher level of anti-*H. pylori* antibodies in SjS patients Increased incidence of mucosal associated lymphoid tissue and lymphomas in parotid and lacrimal glands of SjS patients Against: Low levels of anti-*H. pylori* antibodies in SjS patients compared to controls	Inconclusive
SSc	Support: Higher incidence of *H. pylori* antibodies in SSc patients than controls *H. pylori* eradication improves Raynaud's in SSc patients Possible protective role against Barrett's esophagus Higher level of CagA strain *H. pylori* infected patients Against: Low incidence of anti-*H. pylori* antibodies compared to controls	Inconclusive
RA	Support: Increased rheumatoid factor IgM from B cells chronically stimulated with *H. pylori* urease Against: Low prevalence of anti-*H. pylori* in RA patients Unchanged clinical course or symptomatology after *H. pylori* eradication	Unlikely
SLE	Support: *H. pylori* urease exposure induced anti-ssDNA antibody production in an animal model of SLE Against: Low levels of anti-*H. pylori* found among SLE patients, at levels comparable to controls Negative association between *H. pylori* seropositivity and the development of SLE in African-American women	Unlikely
ITP	Support: Improvement of platelet counts following *H. pylori* eradication (CagA type *H. pylori* in particular) Anti-CagA antibodies cross-react with peptides on platelets of ITP patient Platelet associated IgGs declined following *H. pylori* eradication Found in high prevalence in some ITP cohorts Platelet eluates from ITP patients recognize *H. pylori* CagA Against: Low levels of *H. pylori* found in ITP patients	Probable
AiTD	Support: Higher seropositivity and positive stool cultures for *H. pylori* in Graves' disease patients CagA strain predominant among Graves' disease patients Amino acid similarities between CagA and thyroid peroxidase Reduction in anti-thyroid antibodies following *H. pylori* eradication Against: Low levels of infection among Hashimoto's thyroiditis patients	Probable in Graves' disease Unlikely in Hashimoto's thyroiditis
MS and NMO	Support: High rate of *H. pylori* infection among NMO patients Correlation between *H. pylori* infection and presence of aquaporin-4 antibodies Against: *H. pylori* infection rates in MS patients similar to or lower than control groups	Probable in NMO Unlikely in MS
Psoriasis	Support: Higher levels of anti-*H. pylori* antibodies in patients Appears to be correlation between *H. pylori* infection and disease severity Clinical improvement following *H. pylori* eradication Against: No difference in anti-*H. pylori* levels compared to controls No difference of CagA seropositivity between patients and controls	Probable
Behçet's disease	Support: Higher infection prevalence in patients Some clinical improvement noted after eradication Against: No difference between patients and controls	Unlikely
Alopecia areata	Support: Higher infection prevalence Against: No difference in infection prevalence between patients and controls	Unlikely

PBC	Support: Higher prevalence of anti-*H. pylori* antibodies among PBC patients Amino acid similarities between pyruvate dehydrogenase E2 (PDC-E2) and urease beta of *H. pylori* Against: No differences of infection found between patients and controls No immunological cross reactivities at the B or CD4 T-cell level No crossreactivity between *H. pylori* VacA and PDC-E2	Unlikely
AIH	Support: No current evidence Against: No differences in anti-*H. pylori* antibodies between patients and controls No significant difference between *H. pylori* in liver tissues in patients compared to controls	Unlikely
PSC	Support: Detectable *H. pylori* DNA in PSC liver samples CagA in samples from PSC patients Concomitant ulcerative colitis may be related to *H. pylori* translocation from the gut to the liver Against: No difference in *H. pylori* prevalence among pediatric or adult PSC patients compared to controls No significant difference between *H. pylori* in liver tissues in patients compared to controls	Unlikely

Helicobacter pylori (*H. pylori*) has been implicated in the development of several autoimmune diseases. This table summarizes some of the evidence in support or against this hypothesis in various autoimmune diseases. Overall opinions reflect an inconclusive evidence base, those which are unlikely, and those which have a relatively strong or strong (probable) evidence base. SjS: Sjogren's syndrome; SSc: Systemic sclerosis; RA: Rheumatoid arthritis; SLE: Systemic lupus erythematosus; ITP: Immune thrombocytopenic purpura; AiTD: Autoimmune thyroid disease; MS: Multiple Sclerosis; NMO: Neuromyelitis optica; PBC: Primary biliary cirrhosis; AIH: Autoimmune hepatitis; PSC: Primary sclerosing cholangitis.

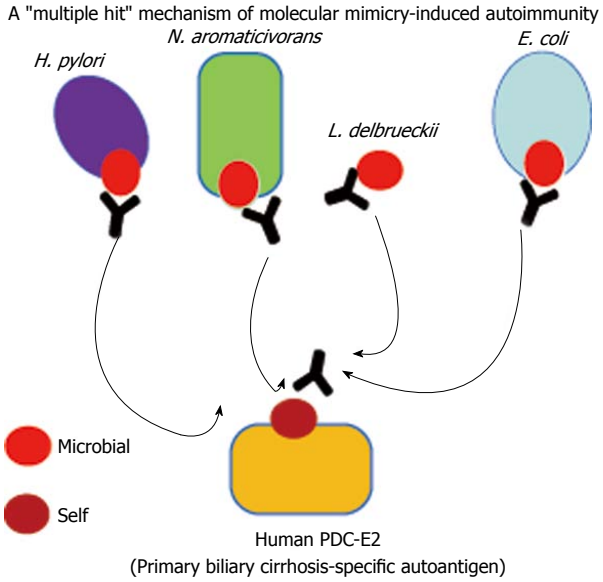

Figure 1 A "multiple hit" molecular mimicry mechanism involving microbial mimics originated from *Helicobacter pylori* and other microbes linked with primary biliary cirrhosis. The major autoepitope of primary biliary cirrhosis-specific anti-mitochondrial antibodies (PDC-E2, pyruvate dehydrogenase complex) shares amino acid similarities with 4 microbial mimics from *Helicobacter pylori* (*H. pylori*)[142], *N. aromaticivorans*[154], *L. delbrueckii*[155,156], and *E. coli*[140,157,158]. The working hypothesis is that exposure of susceptible individuals to infections caused by these microbial agents will initiate humoral and cellular immune responses against microbial epitopes (in our case, these will be those sharing similarity with the self-epitope). Antibodies or T-cells against the microbial mimics may then cross-react with the human autoepitope initiating an autoreactive immune response which could lead to the induction of cellular damage and the perpetuation of autoimmunity (and can cause autoimmune disease). Experimental data so far provided demonstrate the existence of cross-reactive responses between self and microbial peptides from *E. coli, N. amoraticivorans,* and *L. delbrueckii.* However, experimental testing has shown that the *H. pylori* mimic (from urease beta) is not a target of cross-reactive responses specifically present in primary biliary cirrhosis[159]. The prevailing notion is that the mimic from *H. pylori* does not share amino acid similarity to an extent that could initiate cross-reactive response. On the contrary, the other microbial mimics have sufficient homologies with the human autoepitope and can promote molecular mimicry-based immune responses against self.

be autoimmune (such as chronic fatigue syndrome) have not been evaluated for *H. pylori* involvement.

H. pylori is one of the very few infectious agents (along, for example, with Epstein-Barr virus) that have been considered a common denominator in more than 30 autoimmune disorders (Figure 1). Most research in this area has been limited to serological studies investigating two main topics: first, the prevalence of *H. pylori* in the disease under investigation *vs* the control groups; and second, the extent by which *H. pylori* eradication improves the symptomatology of the patients. However, both approaches suffer from conceptual and design constraints. For example, serological studies investigating the prevalence of anti-*H. pylori* antibodies in patients and controls have so far provided discrepancies. Demographic details which are known to affect *H. pylori* status must also be taken into account in cohort selection. This approach will help us to understand whether *H. pylori* infection predisposes to (or protects from) the development of specific autoimmune diseases. Also, the fact that the prevalence of *H. pylori* infection does not differ amongst diseases and control groups does not necessarily mean that this pathogen does not play an important role in the development of immune-mediated disease. Thus, several investigators have considered that it is not the infection per se but the ability of susceptible individuals to mount an immune response against hsps or other immunologically-important *H. pylori* antigens that plays a permissive role in the loss of immunological tolerance to self-antigens. A possibility also exists that the pathogen exerts its pathogenic effects in a "hit-a-run" scenario, (*i.e.*, long after the inflammation caused by the original infection). This could make it almost impossible to link the disease with the microbe in

biological material from individuals already suffering from the disease and its unwanted complications. Longitudinal studies enrolling patients at very early stages of the disease may help us to address this issue. For example, relevant autoantibodies may appear years before clinical manifestations of RA or SLE present. Researchers must also take into account reports indicating that infection with this pathogen may indeed confer protection rather than susceptibility to the development of autoimmunity.

Another topic which needs to be addressed is that the eradication of other autoimmune disease-relevant microbial agents is responsible for the improvement of symptoms of the patients receiving eradication therapy for *H. pylori*. In addition, *H. pylori* eradication may alter the microbiome status of the infected individuals, possibly promoting the persistence of potent infectious inducers of autoimmunity[5]. An immunosuppressive effect of medication may be another possibility. These hypotheses need to be addressed experimentally. Also, work on animal models of diseases and the role of infection with this pathogen are scarce. It is therefore apparent that the role of *H. pylori* in the development of autoimmune disease needs further research, as positive findings may indicate the need for eradication of the pathogen to alter the clinical course, or prevent autoimmune disease in those at risk.

In conclusion, *H. pylori* remains one of the most attractive candidate pathogens that could trigger autoimmunity. The ubiquitous nature of this pathogen may explain why it has been implicated in a large number of autoimmune conditions. There is no doubt that more basic work in immunological aspects of the microbial-host interactions is needed to address the pathogenic role of this multi-faceted pathogen.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (6): *Helicobacter pylori*

Helicobacter pylori gamma-glutamyl transpeptidase and its pathogenic role

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Abstract

Helicobacter pylori (*H. pylori*) gamma-glutamyl transpeptidase (GGT) is a bacterial virulence factor that converts glutamine into glutamate and ammonia, and converts glutathione into glutamate and cysteinylglycine. *H. pylori* GGT causes glutamine and glutathione consumption in the host cells, ammonia production and reactive oxygen species generation. These products induce cell-cycle arrest, apoptosis, and necrosis in gastric epithelial cells. *H. pylori* GGT may also inhibit apoptosis and induce gastric epithelial cell proliferation through the induction of cyclooxygenase-2, epidermal growth factor-related peptides, inducible nitric oxide synthase and interleukin-8. *H. pylori* GGT induces immune tolerance through the inhibition of T cell-mediated immunity and dendritic cell differentiation. The effect of GGT on *H. pylori* colonization and gastric persistence are also discussed.

Key words: *Helicobacter pylori*; Gamma-glutamyl transpeptidase; Bacterial virulence factor; Gastric epithelial cell damage; T cell-mediated immunity

Core tip: In this review, we focus on the biochemical features and physiological role of *Helicobacter pylori* (*H. pylori*) gamma-glutamyl transpeptidase and analyze the mechanisms through which gamma-glutamyl transpeptidase affects *H. pylori* gastric colonization, persistence, immune tolerance and damage to the gastric mucosa.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a gram-negative, microaerophilic, S-shaped bacterium that colonizes approximately 50% of the world's population. *H. pylori* infection causes chronic gastritis, which is asymptomatic in the majority of carriers but may evolve into more severe disease, such as atrophic gastritis, gastric and duodenal ulcers and mucosa-associated lymphoid tissue lymphoma and gastric adenocarcinoma[1]. *H. pylori*-induced gastroduodenal disease depends on the inflammatory response of the host and on the production of specific virulence factors, such as urease, which is responsible for ammonia generation;

Table 1 Reported *Helicobacter pylori* gamma-glutamyl transpeptidase effects

Effects	Ref.
Involved in *H. pylori* colonization and persistence in the gastric mucosa	[5,6]
Hydrolysis of extracellular glutamine and glutathione to generate glutamate that is transported into the *H. pylori* cell	[8]
Highly active periplasmic deamidase involved in ammonia production	[8,20]
Significantly higher GGT activity in strains obtained from patients with peptic ulcer disease	[21]
Gastric epithelial cell death - Mitochondria-mediated apoptosis in gastric epithelial cells	[7,16,24]
Cell-cycle arrest of gastric epithelial cells	[24]
Glutathione degradation-dependent gastric epithelial cell death	[8,27]
H_2O_2 generation, nuclear factor-κB activation and interleukin-8 production in gastric epithelial cells	[21,27]
Induction of EGF-related growth factors and COX-2 in gastric epithelial cells	[15]
Induction of apoptosis and inflammation in human biliary cells	[25]
Degradation of the apoptosis-inhibiting protein survivin in gastric epithelial cells	[30]
Inhibition of T cell proliferation and induction of G1 cell cycle arrest	[9-11]
Induction of microRNA-155 in human T cells	[38]
Gastric persistence and immune tolerance	[12]

H. pylori: *Helicobacter pylori*; GGT: Gamma glutamyl transpeptidase; EGF: Epidermal growth factor; COX-2: Cyclooxygenase 2.

the vacuolating cytotoxin VacA; the cytotoxin-associated gene A product CagA; and the type Ⅳ secretion system encoded by the *cag* pathogenicity island[1-4]. Another virulence factor, gamma-glutamyl transpeptidase (GGT), has been shown to play a role in the colonization of the gastric mucosa by *H. pylori*[5,6], to induce the apoptosis of gastric epithelial cells[7,8], and to inhibit T cell proliferation and dendritic cell differentiation[9-12] (Table 1).

In this review, we focus on the biochemical features and physiological role of *H. pylori* GGT and analyze the mechanisms through which GGT plays a role in *H. pylori* infection, gastric persistence, immune tolerance and gastric mucosa damage.

BIOCHEMICAL FEATURES AND PHYSIOLOGICAL ROLE OF *H. PYLORI* GGT

GGT is a threonine N-terminal nucleophile (Ntn) hydrolase that catalyzes the transpeptidation and hydrolysis of the gamma-glutamyl group of glutathione and related compounds[13]. GGT is widely distributed in living organisms and is highly conserved, with mammalian and bacterial homologs often sharing more than 25% of their sequence identity[14]. GGT is found in all gastric *Helicobacter* species, but among the enterohepatic *Helicobacter* species, it is found only in *H. aurati*, *H. bilis*, *H. canis*, *H. muridarum* and *H. trogontum*[5,7,15,16]. The biochemical features of *H. pylori* GGT and its physiological role are summarized in Figure 1.

H. pylori GGT is synthesized as a 60 kDa proenzyme that autocatalytically forms a heterodimer of 40 and 20 kDa subunits[5,7,14,15]. Threonine380 at the N-terminus of the small subunit is the cleavage site, and it is required for the protein's autocatalytic activity[14]. The enzymatic activity of the protein resides in the small subunit with the gamma-glutamyl binding site at the Tyr433 residue, and the Arg475 residue and the C-terminus of 20 kDa subunit contribute to catalysis[17,18]. *H. pylori* GGT possesses a signal peptide and has been isolated by two independent research groups as a secreted protein in bacterial broth culture filtrates[15,19]. Nevertheless, another research group identified *H. pylori* GGT as a periplasmic protein that is likely to associate with the membrane by ionic bonds[7].

Purified *H. pylori* GGT exhibits hydrolysis activity with very high affinities for glutamine and glutathione. *H. pylori* GGT converts glutamine into glutamate and ammonia, and converts glutathione into glutamate and cysteinylglycine, through hydrolysis[8]. Because *H. pylori* cells are unable to directly take up extracellular glutamine and glutathione, these substances are hydrolyzed into glutamate through the action of GGT, either as a secreted or periplasmic enzyme. These results indicate that the main physiological role of *H. pylori* GGT is to enable bacterial cells to use extracellular glutamine and glutathione as sources of glutamate. The resulting glutamate is then transported by a Na^+-dependent reaction into *H. pylori* cells, where it is primarily incorporated into the TCA cycle and partially used as a substrate for glutamine synthesis[8]. *H. pylori* GGT also has a physiological roles as a periplasmic deamidase and as a contributor with asparaginase to the extracellular production of ammonia[8,20]. The ammonia produced by *H. pylori* GGT can be used as a nitrogen source for bacterial cells and for resisting the acidic gastric environment. The extracellular production of ammonia, along with the consumption of extracellular glutathione and glutamine, may alter the redox balance of host cells in the gastric mucosa and render the host cells more sensitive to the toxic effects of reactive oxidizing substances, which in turn cause DNA damage and apoptosis (see below). The physiological roles exerted by *H. pylori* GGT in bacterial cells and in the host cells could provide metabolic advantages during the establishment of *H. pylori* infection. In fact, previous studies have shown that *H. pylori* GGT plays an important role in the bacterial colonization of the gastric mucosa, and *H. pylori* GGT-defective isogenic strains are unable to colonize[5] or are less efficient[6] at colonizing the gastric mucosa of mice or piglets.

EFFECTS OF *H. PYLORI* GGT ON GASTRIC EPITHELIAL CELLS

Virulence can be defined as the ability of a pathogen to damage its host[3]. Although virtually all wild-type *H. pylori* strains produce GGT, strain-to-strain variations in GGT

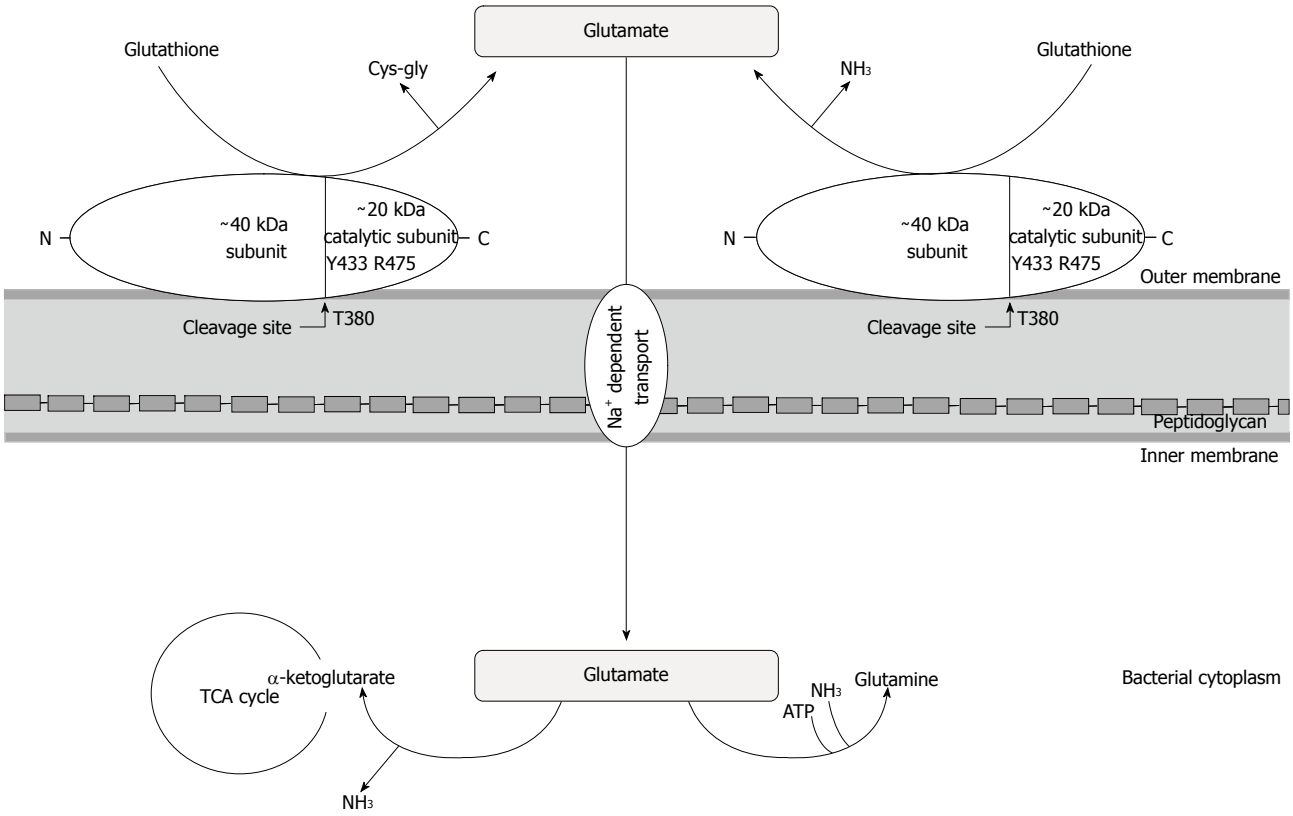

Figure 1 Biochemical features and physiological role of *Helicobacter pylori* gamma-glutamyl transpeptidase. *Helicobacter pylori* (*H. pylori*) gamma-glutamyl transpeptidase (GGT) is a secreted protein of 40 and 20 kDs subunits that converts glutamine to glutamate and ammonia, and glutathione to glutamate and cysteinylglycine. The glutamate produced is then transported into *H. pylori* cells, where it is incorporated into the tricarboxylic acid (TCA) cycle or utilized for glutamine synthesis.

level have been demonstrated among clinical isolates from patients with different disease statuses[21]. In particular, a significantly higher GGT activity has been observed in *H. pylori* isolates obtained from patients with peptic ulcer disease relative to those obtained from patients with nonulcer dyspepsia[21]. Thus, there is evidence of a direct relationship between GGT production and the development of more severe gastroduodenal diseases. This finding stresses the clinical relevance of GGT as a virulence factor in the overall *H. pylori*-induced pathogenic action. That gastric ulcer is associated with a high risk of gastric cancer[1] suggests that GGT may play an important role in *H. pylori*-induced carcinogenesis.

By favoring *H. pylori* colonization of the gastric mucosa[5,6], likely through its lymphocyte-inhibiting action[10] (see below), GGT might also act indirectly by allowing other independent virulence factors (such as CagA, VacA, *etc.*) to better exert their damaging actions against the gastric mucosa. Nevertheless, mounting evidence suggests that GGT exerts a direct damaging effect on gastric epithelial cells. The effects of *H. pylori* GGT on gastric epithelial cells are summarized in Figure 2.

Apoptosis-related effects

In 2003, Shibayama *et al*[7] demonstrated that purified *H. pylori* GGT was able to cause apoptosis in cultured gastric epithelial cells (AGS cell line) in a dose-dependent manner. This proapoptotic activity was strictly dependent on *H. pylori* GGT enzymatic activity, which was completely blocked by incubation with a glutamine analog that binds and inhibits GGT and other glutaminases. It is well-known that *H. pylori* infection induces apoptosis in gastric epithelial cells[22]. An increase in apoptosis may play a significant role in the development of pathological outcomes by disturbing the balance between the rate of new cell production and the rate of cell loss, with atrophic gastritis and gastric dysplasia (*i.e.*, gastric preneoplastic lesions) being associated with an increased rate of apoptosis[22]. Interestingly, Shibayama *et al*[7] also observed that GGT induced necrosis rather than apoptosis in a different gastric epithelial cell line (*i.e.*, KATO Ⅲ). A similar difference in the type of cell death induced among different experimental models has recently been observed for another *H. pylori* virulence factor, VacA[23]. This finding raises the key question of how and to what extent the *in vitro*-derived findings really mimic the *in vivo* situation[2]. Unlike apoptosis, necrosis results in the release of proinflammatory proteins, thereby augmenting gastric mucosal inflammation and contributing to the pathogenesis of peptic ulceration and gastric cancer[3,23].

In vitro, GGT-induced apoptosis has been shown to occur *via* the so-called "intrinsic" (*i.e.*, mitochondria-dependent) pathway with the release of cytochrome *c* in the cytosol and the activation of caspase-9 and -3. These caspases are critical components of the apoptotic machinery and are associated with the up-regulation of proapop-

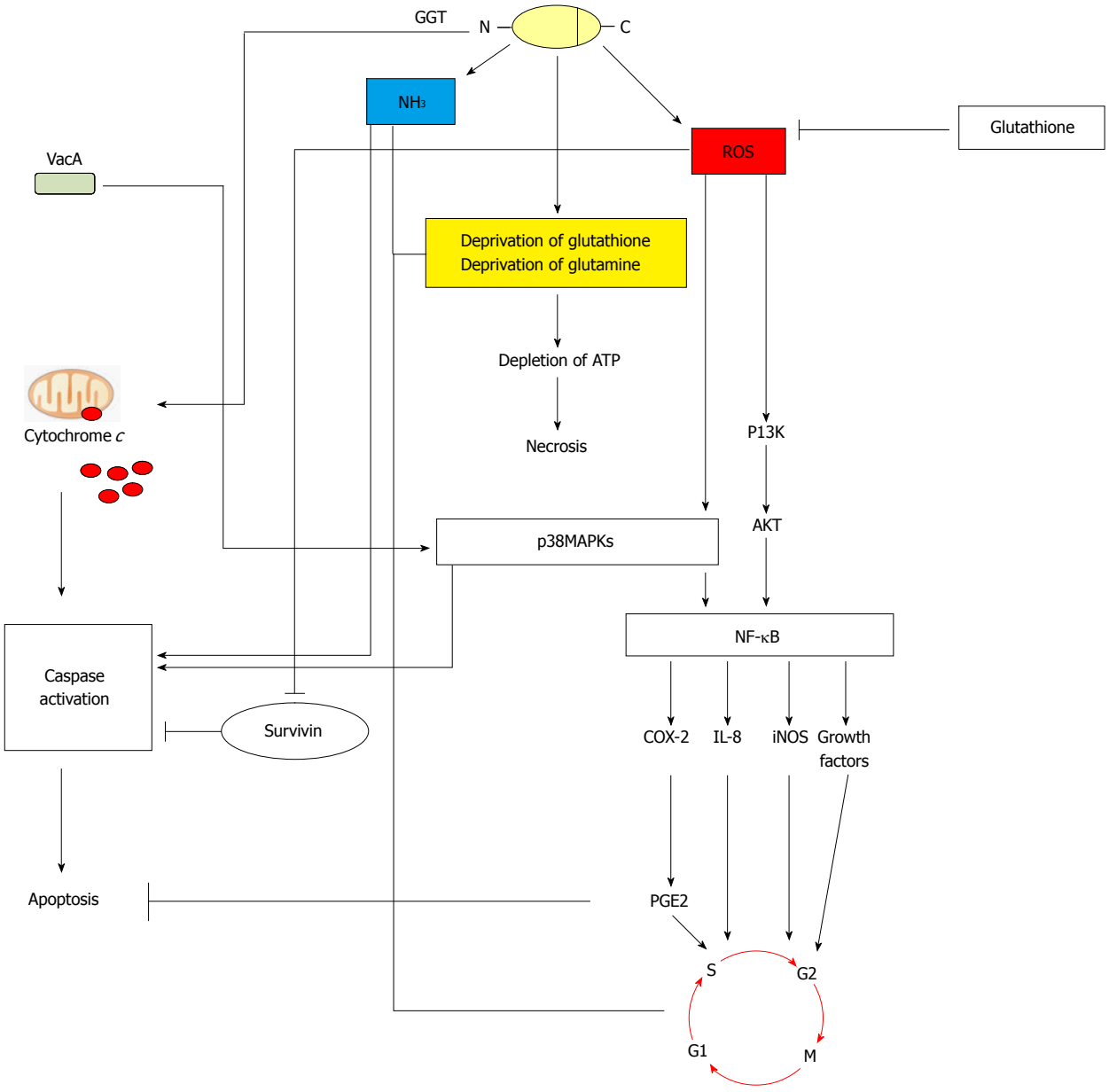

Figure 2 Effects of *Helicobacter pylori* gamma-glutamyl transpeptidase on gastric epithelial cells. *Helicobacter pylori* (*H. pylori*) gamma-glutamyl transpeptidase (GGT) causes consumption of mucosal glutamine and glutathione, production of ammonia and generation of ROS. These products induce caspase activation and apoptosis, ATP-depletion and necrosis, and cell-cycle arrest at G1-S phase in gastric epithelial cells. The effect of vacuolating cytotoxin (VacA) on caspase activation and apoptosis of gastric epithelial cells is also shown. *H. pylori* GGT may also inhibit apoptosis and induce proliferation through p38 MAPKs, AKT and NF-κB activation and subsequent COX-2, iNOS, growth factors and interleukin-8 (IL-8) induction. ROS: Reactive oxygen species; p38 MAPK: p38 mitogen-activated protein kinase; PI3K: Phosphatidylinositide 3-kinase; AKT: AKT kinase; NF-κB: Nuclear factor κB; COX-2: Cyclo-oxygenase 2; iNOS: Inducible nitric oxide synthase; PG: Prostaglandin.

totic members of the Bcl-2 protein family (such as Bax) and the downregulation of antiapoptotic proteins of the same family (Bcl-2 and Bcl-xL)[24]. It is worth noting that similar results have been found recently using human cholangiocarcinoma cells (KKU-100 cell line) as an *in vitro* cell model, in which *H. pylori* GGT was also found to increase both the level of *iNOS* gene expression and the secretion of interleukin (IL)-8[25]. Based on these results, Boonyanugomol *et al*[25] suggested that *H. pylori* GGT might be involved in the development of hepatobiliary tract cancer by altering cell kinetics and promoting biliary cell inflammation. However, this intriguing hypothesis remains highly speculative given that, as stressed above, the *in vivo* biological plausibility and clinical counterpart of the *in vitro* findings are still far from being confidently ascertained.

Apoptosis-independent antiproliferative effects

Another research group found that recombinant *H. pylori* GGT showed an apoptosis-independent inhibitory effect on AGS cell proliferation in a dose-dependent manner, although the minimum required protein concentration was 25 times higher than the concentration needed to inhibit the proliferation of human T cells[16]. The dis-

crepancies between these results and those of Shibayama *et al*[7] have tentatively been attributed to the different methodologies. In particular, only stress conditions such as serum starvation seem to sensitize AGS cells to GGT-dependent apoptosis[16]. Kim *et al*[26] investigated the effect of *H. pylori* GGT on cell cycle regulation of AGS cells in serum-containing medium. Although the changes were less marked than those in serum-deprived cells, the investigators confirmed the previously observed apoptotic action of GGT and found, in addition, that GGT caused cell cycle arrest at the G1-S phase transition[26]. Cell cycle arrest was associated with altered expression of specific cell cycle regulatory proteins, namely the down-regulation of cyclin E, cyclin A, cyclin-dependent kinase (Cdk) 4 and Cdk 6, and the up-regulation of the Cdk inhibitors p27 and p21. Thus *H. pylori* GGT seems to act as a brake at the G1 to S phase transition, thereby disrupting the normal function of several components of the cell cycle which also lead to apoptosis[26].

GGT-activated molecular pathways in gastric epithelial cells

The mechanisms by which the enzymatic activity of *H. pylori* GGT leads to gastric epithelial cell damage have been carefully investigated by several groups[8,15,20,27]. In mammalian cells, glutathione is synthesized in the cytosol where it reaches mM levels and functions as a redox buffer to detoxify oxidizing molecules. Glutathione may be translocated out of cells, where it serves as a substrate for mammalian cell GGT that is integrated into the plasma membrane using its active site. Because of GGT, the gamma-glutamyl moiety of glutathione is transferred to other amino acids along with the formation of gamma-glutamyl amino acids to be subsequently taken up by the cell; this sequence of events is the so-called "gamma-glutamyl cycle". Because the K_m for the hydrolysis reaction catalyzed by *H. pylori* GGT is much lower than that of the reaction catalysed by human GGT, gastric epithelium colonization by *H. pylori* would result in the exhaustive hydrolysis of epithelial cell glutathione[8]. If either the glutathione supply or its synthesis fails to compensate for its *H. pylori* GGT-dependent hydrolysis, the redox balance of the gastric cell will be impaired. The reduced cytosolic concentration of glutathione makes the epithelial cells more sensitive to the toxic effects of oxidizing molecules, making them more prone to DNA damage, cell cycle alterations, apoptosis and carcinogenesis. Moreover, because glutathione synthesis is an ATP-dependent process, its enhanced degradation by *H. pylori* GGT would also cause increased compensatory energy consumption by the epithelial cells, which in turn would result in impaired cell viability and proliferation. The hydrolytic activity of *H. pylori* GGT also exhibits a very high affinity for glutamine, an important nutrient for the gastric mucosa. Extracellular glutamine depletion by bacterial GGT at the *H. pylori* colonization site would result in the impairment of both the cytoprotective properties of gastric epithelial cells and the immune function of recruited inflammatory cells, for which glutamine is an important respiratory fuel source[8]. In addition, GGT-dependent glutamine hydrolysis is associated with the production of ammonia[8,20], which is well-known not only for its high toxicity to human cells[28] but also for greatly increasing the cytotoxic action of another pivotal *H. pylori* virulence factor, namely the VacA toxin[29].

Flahou *et al*[27] recently confirmed that incubating AGS with *H. pylori* GGT resulted in cell apoptosis. However, they also observed that the supplementation of GGT-treated cells with glutathione strongly enhanced the degree of cell death and resulted in the induction of oncosis/necrosis and not apoptosis. This effect was preceded by increased extracellular H_2O_2 concentrations, which caused lipid peroxidation. These authors concluded that the GGT-mediated degradation of glutathione results in the generation of pro-oxidant products, in turn leading to epithelial cell death, which will be caused by apoptosis or necrosis depending on the amount of extracellular glutathione available as GGT substrate[27]. Indeed, the type of *in vitro* H_2O_2-induced cell death is known to depend on the concentration of this reactive oxygen species (ROS), with the higher concentrations inducing necrosis rather than apoptosis[27]. Like mammalian GGTs, *H. pylori* GGT-mediated extracellular glutathione catabolism produces ROS (such as H_2O_2) by thiol-dependent iron reduction, and this production is increased with the addition of exogenous Fe^{3+} and, conversely, inhibited by treatment with the iron chelator desferrioxamine[21]. Interestingly, it was recently observed[30] that this type of GGT-dependent pathway seems to play a key role in the *H. pylori*-induced loss of the apoptosis-inhibiting protein survivin in gastric epithelial cells by triggering enhanced proteasomal degradation of the protein. The loss of survivin may thus contribute to the increased cell death induced by *H. pylori* GGT.

As demonstrated both in AGS gastric cancer cells and in primary non-transformed gastric epithelial cells, the increased production of H_2O_2 by *H. pylori* GGT also leads to the activation of nuclear factor-κB and the up-regulation of IL-8 which is known to play a major role in the inflammation-associated mucosal injury induced by *H. pylori*[21]. Gong and coworkers[21] also found that *H. pylori* GGT caused oxidative DNA damage, which can be counteracted by preincubation with the H_2O_2 inhibitor N-acetylcysteine, suggesting a key role for H_2O_2 generation in GGT-dependent DNA damage. However, Toller *et al*[31] found that GGT was apparently not involved in DNA double-strand breaks caused by *H. pylori* in primary and transformed murine and human epithelial/mesenchymal cells, suggesting that *H. pylori* GGT did not contribute to the genetic instability and chromosomal aberrations observed during gastric carcinogenesis.

Upregulation of EGF-related peptides and COX-2

The molecular cross-talk between *H. pylori* and human gastric mucosa leading to gastric inflammation and cancer involves also the increased expression of epidermal

growth factor (EGF)-related peptides and the activation of the EGF receptor signal transduction pathway as well as upregulation of the expression of cyclooxygenase (COX)-2, the inducible isoform of the enzyme responsible for prostaglandin production[1,2,15]. Our group[15] demonstrated that GGT is the virulence factor responsible for the *in vitro* up-regulation of both EGF-related peptides and COX-2 in human gastric epithelial cells. This finding was supported by observations showing that all such effects were counteracted by the selective GGT inhibitor acivicin and that an *H. pylori* isogenic mutant strain defective in GGT did not exert any effect on either EGF-related peptides or COX-2 expression[15]. Apparently, a common signal transduction pathway that relies on the activation of phosphatidylinositol-3 kinase and p38 kinase, but not MAP kinase kinase, triggers the GGT-dependent effects on the cell expression of both EGF-related peptides and COX-2. Notably, the GGT-induced up-regulation of EGF-related peptides and COX-2 mRNA expression was significantly inhibited by treatment with desferrioxamine, which inhibits the formation of ROS generated by cysteinylglycine in the presence of transition metals[15]. This last finding suggests that *H. pylori* GGT may trigger a proinflammatory and procarcinogenic mucosal response *via* oxidative stress in gastric mucosal cells.

EFFECTS OF *H. PYLORI* GGT ON T CELL-MEDIATED IMMUNITY

Mounting evidence indicates that *H. pylori* GGT may modulate T cell-mediated immunity and contribute to immune evasion during *H. pylori* infection. Gerhard *et al*[9] first demonstrated that the inhibition of T cell proliferation by *H. pylori* is mediated by a low-molecular weight protein secreted by the bacterium. The same research group identified *H. pylori* GGT as the secreted bacterial protein that induces cell cycle arrest in the G1 phase of T cells and suppresses T cell proliferation[10]. They also identified the disruption of Ras- but not PI3K-dependent signaling by *H. pylori* GGT as the cause of the G1 arrest, and it also suppressed T cell proliferation[10].

VacA toxin has also been identified as an additional bacterial virulence factor that can efficiently block T cell proliferation by inducing G1/S cell cycle arrest[32,33] and inhibiting the activation of nuclear factor of activated T cells (NFAT), a transcription factor acting as a global regulator of immune response genes[32,34]. Interestingly, impairment of the mitochondrial function has been suggested as an additional mechanism involved in the VacA-induced blockade of $CD4^+$ T cell proliferation[35]. A similar action in the T cell mitochondria might also be hypothesized for GGT, accounting for its proven capacity to damage epithelial cell mitochondria. VacA and GGT released from the bacteria in the gastric mucosa may directly contact intraepithelial T cells or penetrate the mucosa-associated lymphoid tissue (MALT) through the opening of tight junctions brought about by *H. pylori*[36]. Notably, *H. pylori* has also been demonstrated to be able to penetrate the gastric epithelium *in vivo* reaching the underlying lamina propria where it directly contacts immune-inflammatory cells[37].

Because *H. pylori* is a cholesterol auxotroph and needs to extract this nutrient from host cells, the inhibitory effects of VacA and GGT on the proliferation of human $CD4^+$ T cells is also modulated by the ability of *H. pylori* to form cholesterol alpha-glucosides[11]. In further support of the roles of VacA and GGT on the inhibition of T cells, it has recently been demonstrated that VacA and *H. pylori* GGT positively regulate the expression of the non-protein-coding microRNA (miRNA) miR-155 and the master T cell regulator Foxp3 in human lymphocytes through a cAMP-dependent pathway[38].

Both VacA and GGT from *H. pylori* may also affect T cell activity in an indirect manner by reprogramming dendritic cells to promote the differentiation of naive T cells into T regulatory (Treg) cells[12]. Treg cell differentiation in response to *H. pylori* infection requires the direct interaction of naive T cells with "tolerogenic" dendritic cells that have been exposed to *H. pylori*, either in the gastric mucosa or in gastric or mesenteric lymph nodes[39,40]. Dendritic cells that have been exposed to *H. pylori* fail to induce T_H1 and T_H17 type T cell responses *in vitro* and *in vivo*; instead, such dendritic cells preferentially induce the expression of the Treg cell-specific transcription factor FOXP3, the surface marker CD25 and the anti-inflammatory cytokine IL-10 in naive T cells[12]. This action may contribute to gastric persistence and immune tolerance during infection, and it may independently potentiate the evasion of the immune response generated by the apoptosis of human monocytes in the presence of *H. pylori* expressing functional *cag* pathogenicity island[41]. The immune response evasion might also be due to the induction of COX-2 in gastric epithelial cells by *H. pylori* GGT[15], which has been shown to suppress the T_H1 polarization of T cell response to *H. pylori*[42].

The effects of *H. pylori* GGT on T cell-mediated immunity could represent the biological basis of observations in animal models, showing an important role for GGT in *H. pylori* colonization[5,6]. Because *H. pylori* has been classified as a type I carcinogen[1], the inhibition of immune responses caused by *H. pylori* GGT might also be an important factor in the induction of malignant MALT lymphoma and adenocarcinoma of the stomach. The effects of *H. pylori* GGT on T cell-mediated immunity are summarized in Figure 3.

CONCLUSION

H. pylori produces a combination of virulence factors that damage the gastric mucosa and subvert the host immune response to allow persistent colonization of the challenging environment of the human stomach. In this review, we focussed on *H. pylori* GGT, a bacterial protein that inhibits cell proliferation and induces the apoptosis of gastric epithelial cells through different pathways involving ammonia and ROS production. This action may

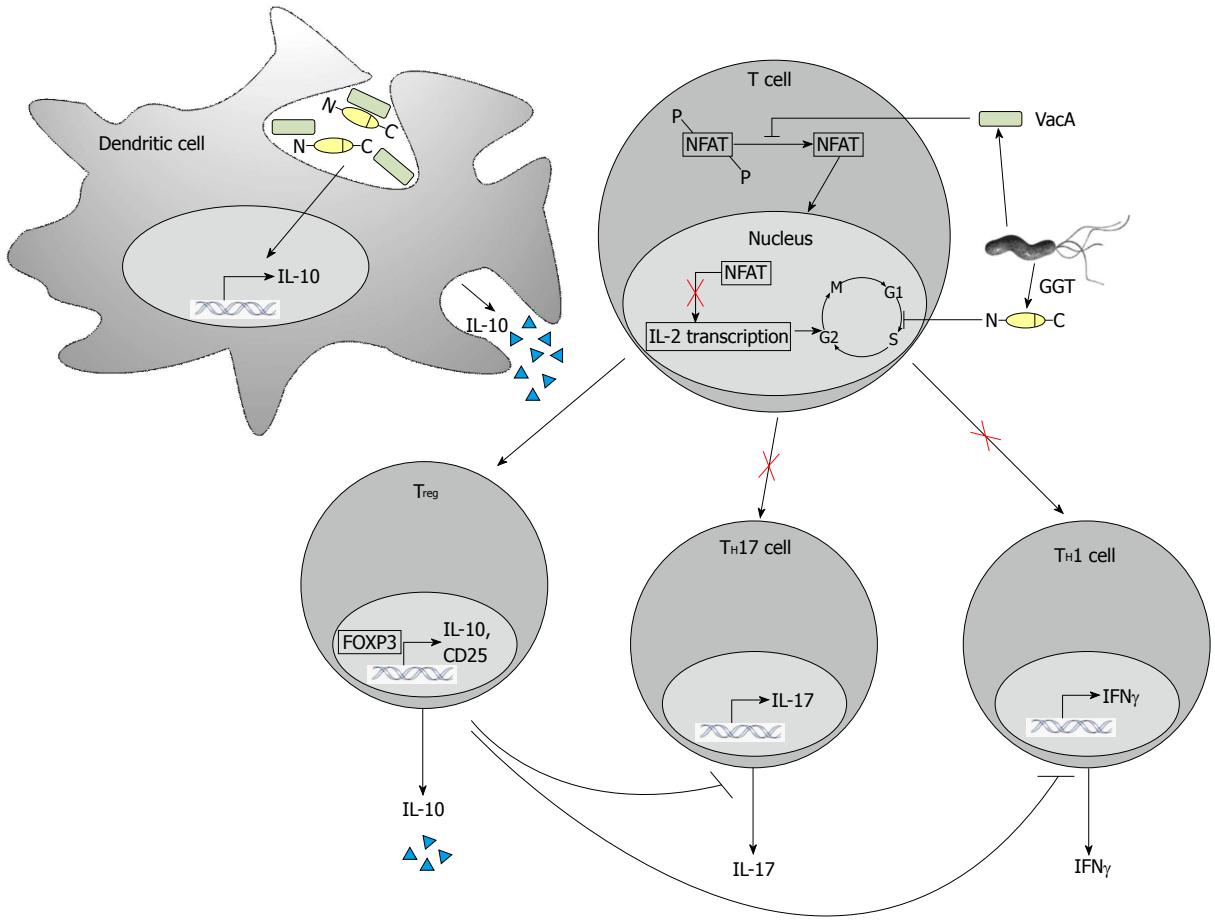

Figure 3 Effects of *Helicobacter pylori* gamma-glutamyl transpeptidase on T cell-mediated immunity. *Helicobacter pylori* gamma-glutamyl transpeptidase (GGT) and VacA inhibit T cell proliferation and differentiation to T helper 1 (T_H1) and T_H17. They also prevent T cell immunity by reprogramming dendritic cells to produce interleukin-10 (IL-10) and IL-18 and promote the differentiation of naive T cells into T regulatory (Treg) cells that further suppress T_H1 and T_H17 effector functions. FOXP3: Forkhead box P3; NFAT: Nuclear factor of activated T cells; IFN-γ: Interferon gamma.

contribute to gastric injury during *H. pylori* infection. Interestingly, *H. pylori* GGT may also stimulate the expression of antiapoptotic factors and factors that protect against cell damage, such as COX-2 and prostaglandins, EGF-related growth factors and iNOS, which could heal gastric mucosa but may also play a procarcinogenic role during infection. The effects exerted by *H. pylori* GGT may depend on the level of GGT expression and/or on the concomitant expression of other bacterial virulence factors. Instead, the effect of *H. pylori* GGT on the inhibition of T cell immunity and dendritic cell maturation may favor colonization and bacterial persistence in the gastric mucosa. The evasion of the immune response by *H. pylori* GGT may also play a role during gastric carcinogenesis. Increased knowledge of the molecular mechanisms underlying *H. pylori* infection may lead to the recognition of potential intervention targets to prevent the progression of chronic gastritis to atrophic gastritis and gastric cancer.

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TOPIC HIGHLIGHT

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Impairment of ghrelin synthesis in *Helicobacter pylori*-colonized stomach: New clues for the pathogenesis of *H. pylori*-related gastric inflammation

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Abstract

Ghrelin, the ligand of growth hormone secretagogue receptor 1a, takes part in several functions of the digestive system, including regulation of appetite, energy homeostasis, gastric acid secretion and motility. Ghrelin has also immunoregulatory properties and is supposed to inhibit some inflammatory pathways that can mediate gastric damage. Interestingly, ghrelin synthesis is reduced in the gastric mucosa of patients with *Helicobacter pylori* (*H. pylori*) infection, a worldwide condition inducing a T helper (Th)1/Th17 cell response-driven gastritis, which may evolve towards gastric atrophy and cancer. In this article, we review the available data on the expression of ghrelin in *H. pylori* infection and discuss how the defective ghrelin synthesis may contribute to sustain the ongoing inflammatory response in this disease.

Key words: Gastritis; Ghrelin; *Helicobacter pylori*; T helper 1 cells

Core tip: The review reports current statements about relationship between gastric ghrelin expression and *Helicobacter pylori* (*H. pylori*) infection. Data present in the literature and emerging from a very recent our study on the anti-inflammatory role of ghrelin and T helper 1 cell response in the stomach during *H. pylori* infection are included.

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GHRELIN, A HORMONE WITH IMMUNOREGULATORY FUNCTIONS

Initially described as a ligand of growth hormone secretagogue receptor 1a (GHS-R1a) expressed by growth hormone-secreting pituitary cells[1], ghrelin is a potent stimulator of growth hormone secretion[2]. Ghrelin is largely produced in the alimentary tract, mainly in the stomach, by gastric X/A-like endocrine cells in rodents and fundic P/D1 cells in humans, while its synthesis gradually diminishes from duodenum to the colon[3]. The large synthesis of ghrelin and expression of GHS-R in the stomach and in other organs and tissues suggested additional effects other than stimulation of growth hormone in the pituitary. Indeed, it is now known that ghre-

lin takes part in several functions (Figure 1), including the regulation of appetite and energy homeostasis, which could favour adiposity and obesity[4-6]. Ghrelin is also produced in pancreas, lung, kidney, testis, placenta and by immune cells[7]. Ghrelin circulates in two major forms, acyl and desacyl ghrelin[8]. Acyl ghrelin has an octanoyl group essential to activate GHS-R1a[9,10-13]. Desacyl ghrelin lacks this octanoyl group and it was early thought to be an inactive form of ghrelin since it does not activate GHS-R1a. Indeed, desacyl ghrelin has been demonstrated to counteract acyl ghrelin and inhibit the stimulation of food intake, gastric and bowel emptying[14,15] and to be involved in several other biological functions (*e.g.*, in the reproductive system, bone metabolism, cardiovascular protection)[16-18]. Ghrelin has been also found to stimulate neurogenesis[19], improve central memory[20], influence sleep-wake cycle[21].

Acylation of ghrelin is mediated by ghrelin-*O*-acyltransferase (GOAT) in both mice and humans[22,23], an enzyme expressed by several tissues, including the stomach and pancreas[15]. Although GOAT expression is high in the stomach, a direct quantitative correlation with the expression of ghrelin mRNA has not been demonstrated[24]. GOAT is also present in the plasma and varies in relationship with the fasting or feeding status[25]. As acylated ghrelin has a short *in vivo* half-life (about 9-13 min)[26], desacyl ghrelin accounts for > 90% of the circulating ghrelin[8] with a ratio of acyl/desacyl ghrelin varying from 1:15 to 1:55[8,27].

Besides physiologic activities, ghrelin exerts a gastroprotective effect during pathological conditions. Indeed, *in vivo* in rats administration of ghrelin attenuates the gastric mucosal lesions induced by detrimental agents, such as ethanol and indomethacin, through an increase of mucosal generation of prostaglandins prostaglandin E2 (PGE2)[28,29]. Ghrelin is also an important regulator of NOS and cyclooxygenase (COX) enzyme systems[1,30-33]. Moreover, studies in different animal models revealed that ghrelin reduces the release of pro-inflammatory cytokines, such as interleukin (IL)-1β, TNF-α, IL-6[34-37] and stimulates the expression of the anti-inflammatory cytokine IL-10[38,39] by T lymphocytes and macrophages in different mechanical or chemical-induced inflammatory conditions. Treatment of human T lymphocytes and monocytes with exogenous ghrelin inhibits the release of pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6[40,41].

Altogether these data underline the gastroprotective functions and the anti-inflammatory role of ghrelin.

HELICOBACTER PYLORI INFECTION ASSOCIATES WITH DECREASED GASTRIC PRODUCTION OF GHRELIN

Helicobacter pylori (*H. pylori*) is a Gram negative microorganism, which colonizes the stomach and causes a chronic gastritis, with the downstream effect of promoting peptic ulcer and cancer[42]. *H. pylori*-related gastritis may progress to atrophy with loss of pyloric and oxyntic glands which, in turn, may negatively affect secretory functions in the stomach. This gastritis related-damage may interfere with ghrelin expression through a loss of P/D1 cells in the fundus and body of the stomach. On the other hand, *H. pylori* could directly act on mechanisms controlling ghrelin production through the release of cytotoxins, lipopolysaccharide (LPS) and other noxious agents[43]. Indeed, ghrelin expression is reduced in gastric biopsies of *H. pylori* uninfected subjects following a 24 h incubation with *H. pylori*-derived culture broth[44]. This evidence is in keeping with the observation[45] that LPS originating from Gram negative bacteria wall, such as *H. pylori*, intraperitoneally administered in rats reduces plasma ghrelin levels during the first three hours post-injection, probably through an interleukin-1-stimulated release of prostacyclin, which acts directly on PGI_2 receptor-expressing ghrelin-producing cells of the gastric oxyntic mucosa.

Many researchers compared circulating values of ghrelin in infected and non-infected patients with inconsistent results. The majority of these studies[46-66] found lower levels of circulating ghrelin in *H. pylori* positive subjects in Asia and Europe but not in United States. Conflicting results were also obtained when the effect of *H. pylori* eradication on ghrelin plasma levels was evaluated[46,56-58,67-72]. A metanalysis by Nweneka and Prentice concluded that circulating ghrelin is significantly lower in *H. pylori*-positive than negative subjects but *H. pylori* eradication does not significantly modify plasma ghrelin levels[73]. Several factors could explain discrepancy in the results, such as gender[53,74], age[75,76], gastric-related diseases (higher levels in gastritis and peptic ulcer[77] than in gastric cancer[78]), *H. pylori* strain differences (different expression of cytotoxyns)[43], extent and severity of gastritis (presence or not of atrophy)[60,79,80] and different immunoassays used to measure ghrelin.

Ghrelin expression in the stomach was also assessed by quantification of the gastric ghrelin peptide content or ghrelin mRNA expression in endoscopic biopsies[44,51,52,58,59,62,65,70,71,80-83]. In all studies but three[51,59,65] lower amounts of ghrelin peptide were found in *H. pylori* infected in respect to non-infected subjects. In contrast to circulating ghrelin, ghrelin mRNA[61,71] and ghrelin immunoreactive cells[83] increased after *H. pylori* eradication.

H. pylori infection has been reported to influence body mass index (BMI), as it seems to be higher in infected patients than non-infected subjects[84]. Several studies[85-88] also reported an increase of BMI following *H. pylori* eradication in Asia and in Europe. How *H. pylori* may influence BMI has not yet been fully understood but the reduction of dyspepsia following *H. pylori* eradication could increase the appetite and consequently body weight[88]. The restoration of gastric ghrelin expression and an increase of circulating ghrelin levels have been thought to be responsible of the weight gain process following *H. pylori* eradication[67]. This hypothesis, however, has not been confirmed in other studies[68,81] in which an increase

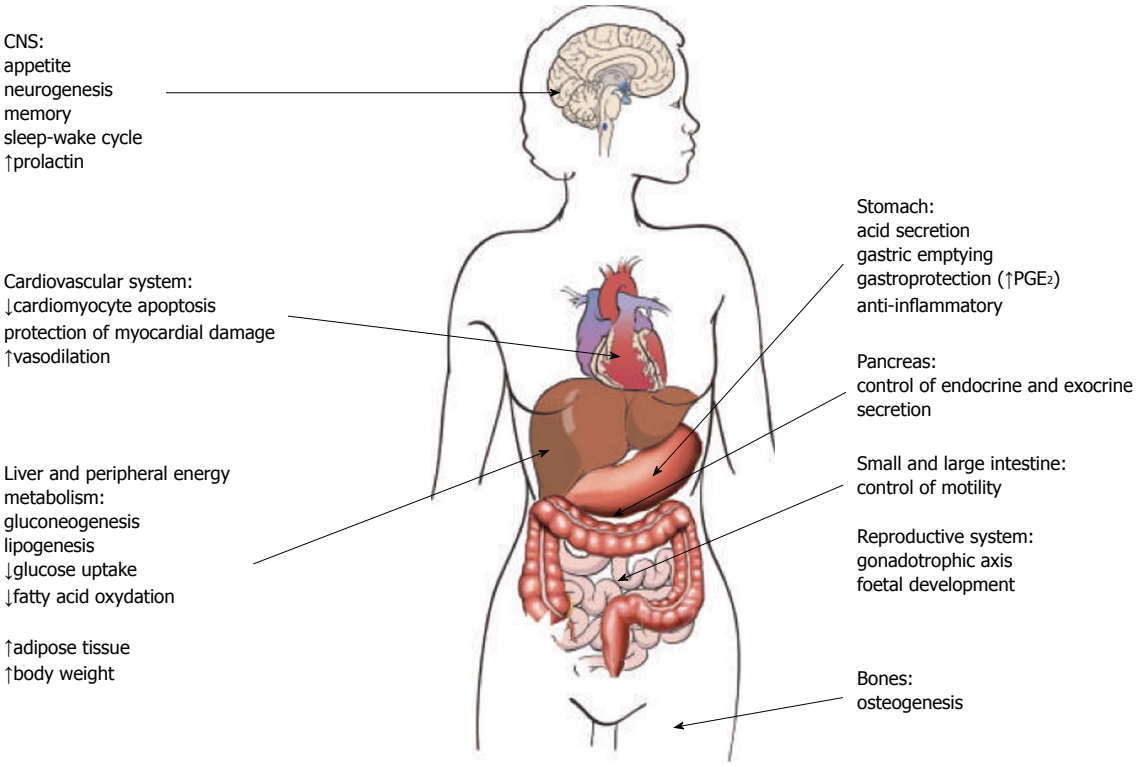

Figure 1 Main functions of ghrelin in the human body. INF: Interferon; IL: Interleukin; PGE_2: Prostaglandin E2; Th1: T helper 1; APC: Antigen presenting cell.

of gastric ghrelin synthesis following *H. pylori* eradication was not correlated to the raise of BMI. Therefore, the exact contribution of ghrelin in the increase of BMI following *H. pylori* eradication remains to be elucidated.

IMPACT OF GHRELIN DOWN-REGULATION ON THE GASTRIC INFLAMMATORY RESPONSE TO *H. PYLORI*

The mechanisms by which *H. pylori* drives the tissue damaging inflammatory response in the stomach have been largely investigated. It is known that the response to *H. pylori* infection and the variable mucosal damage are probably influenced by bacterial and host factors[89,90]. Gastric epithelial cells infected by *H. pylori* exhibit hyperactivation of nuclear factor κB (NF-κB) and produce elevated levels of chemokines, such as IL-8, which contribute to recruit neutrophils into the inflamed tissue[91,92]. *H. pylori*-driven gastritis is associated with a strong activation of T helper (Th)-type 1 cells, which release large amounts of interferon (IFN)-γ[93,94] and express high T-bet, a Th1-inducing transcription factor[95]. Consistently, *H. pylori*-infected biopsies contain elevated levels of IL-12, the main Th1 inducing factor in human beings[96]. The large release of INF-γ leads to several inflammatory responses, including the induction of Smad7[97], a strong inhibitor of transforming growth factor (TGF)-β1 activity[98,99]. The defective TGF-β1 activity documented in *H. pylori*-infected biopsies fits with the demonstration that loss of TGF-β1 in mice associates with a severe gastric inflammation and mucosal damage[100]. Restoring TGF-β1/Smad3 activity with a specific SMAD7 antisense oligonucleotide markedly inhibits Th1 inflammatory cytokine response[97], thus highlighting the importance of the impaired TGF-β1 activity in maintaining the *H. pylori*-driven pathological response. Recently, *H. pylori*-associated gastritis has been demonstrated to be characterised by elevated levels of another subset of Th cells, termed Th17 and producing IL-17A, IL-17F, IL-21, IL-22 and IL-26[101-105]. Collectively these data, together with the demonstration that both Th1 and Th17 cells can be pathogenic in mice infected with Helicobacter species, suggest that *H. pylori* infection may elicit Th1- and Th17-cell immune response in the gastric mucosa thereby contributing to amplify the ongoing mucosal inflammation and favouring the development of gastric lesions. How *H. pylori* infection induces Th1 and TH17 cell response is not, however, fully understood. It is possible that factors released by *H. pylori* stimulate macrophages and dendritic cells which, in turn, produce factors driving Th1 and Th17 responses. This is supported by the evidence that the *H. pylori* neutrophil-activating protein (*H. pylori*-NAP) is able *in vitro* to stimulate IL-12 production via agonistic interaction with toll-like receptor 2 and promote Th1 cell polarization[105]. *H. pylori*-NAP enhances also IL-23, a cytokine involved in the expansion/maintenance of Th17 cell responses. *H. pylori* infection may also down-regulate factors involved in the negative regulation of Th1 and Th17 cell responses.

The *H. pylori*-related impairment of ghrelin synthesis in the stomach could represent another step in the

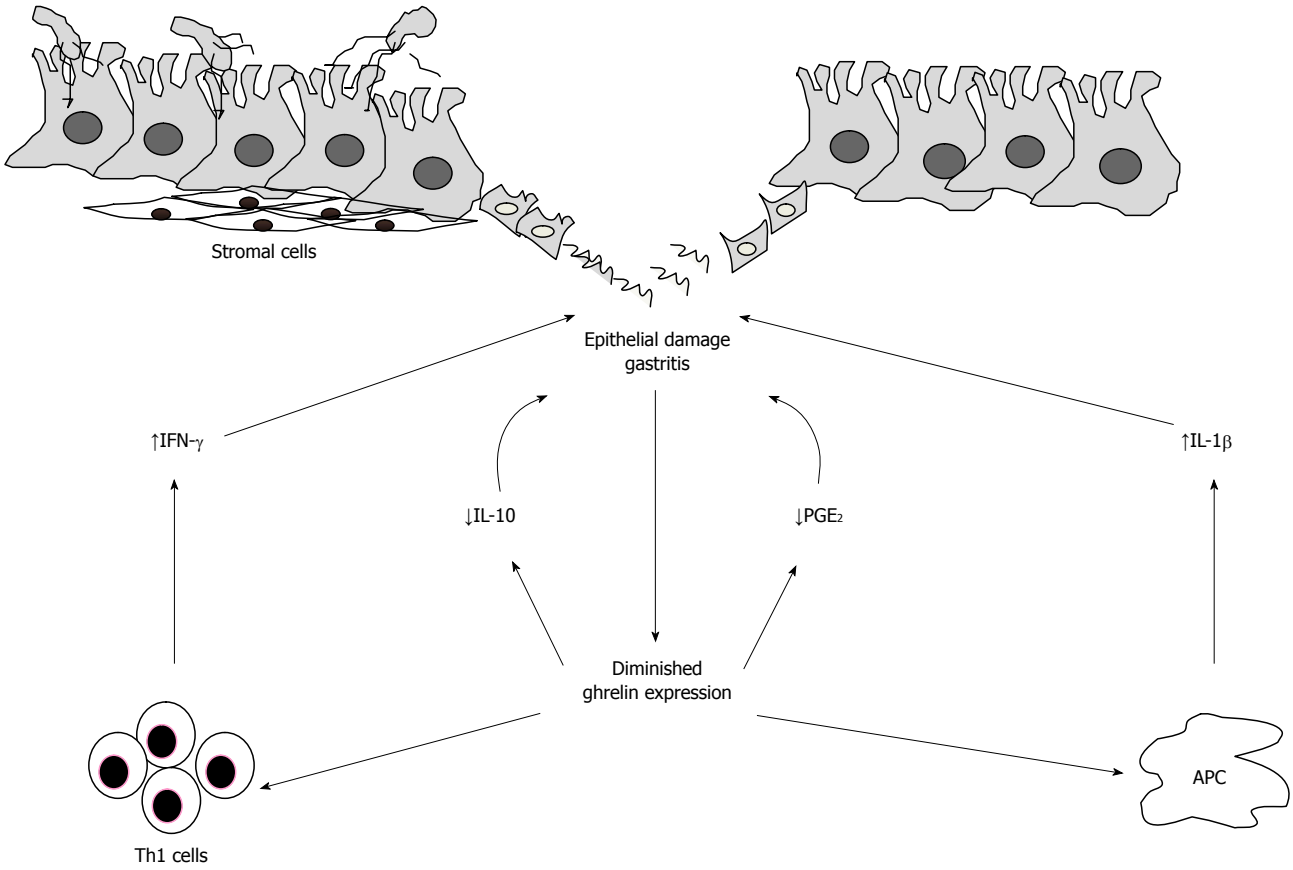

Figure 2 Downregulation of ghrelin expression in the stomach during *Helicobacter pylori* infection. Epithelial damage and gastritis induced by *Helicobacter pylori* determine a diminished expression of ghrelin which, in turn, sustains the ongoing T helper (Th) 1 cells response. Down regulation of ghrelin is also followed by a reduced release of Prostaglandin E2 (PGE_2) and interleukin (IL)-10 which, together with pro-inflammatory factors as IL-1β, contribute to the detrimental immune response and damage in the stomach. APC: Antigen presenting cell.

damaging process caused by the microorganism (Figure 2). We have recently shown that treatment of *H. pylori*-infected human gastric biopsies and lamina propria mononuclear cells isolated from *H. pylori*-colonized gastric biopsies with exogenous ghrelin down-regulated the expression of IFN-γ and IL-12[44]. In contrast, no change in IL-4 was seen following ghrelin treatment, thus indicating that action of ghrelin is confined to Th1 cell immune response. These finding are in line with previous studies showing that ghrelin counteracts other *H. pylori*-induced pathogenic signals, such apoptosis of gastric epithelial cells[33] and activation of important transcription factors, such NF-κB and MAP kinases[106].

The exact role of ghrelin in the development and progression of gastric cancer cells remains to be ascertained. There is evidence that circulating ghrelin levels are deeply diminished in patients with gastric cancer as compared to healthy subjects[78,107], raising the possibility that the diminished ghrelin expression seen in *H. pylori*-positive patients may be involved in the progression of gastric cancer. On the other hand, studies[108,109] with cultured gastric cancer cells have shown that ghrelin may be mitogenic and, therefore, have a promoting effect on neoplastic cell growth.

CONCLUSION

Ghrelin, a hormone with anti-inflammatory and anti-apoptotic properties, is supposed to play an important gastroprotective role. The findings described in this article indicate that *H. pylori* infection associates with a marked down-regulation of ghrelin synthesis, thus delineating a scenario in which such a defect contributes to sustain the *H. pylori*-driven pathogenic response. Further experimentation would be, however, necessary to ascertain the basic mechanism underlying the negative regulation of ghrelin synthesis by *H. pylori* as well as to evaluate whether there are inflammatory pathways which rely strongly on ghrelin down-regulation. Studies are also needed to determine the contribution of the diminished ghrelin production in the evolution of *H. pylori*-associated pathology, as ghrelin can increase the production of PGE2, a protective factor for the gastric mucosa, which has been also implicated in the pathogenesis of cancer[80,109].

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (6): *Helicobacter pylori*

Helicobacter pylori infection in obesity and its clinical outcome after bariatric surgery

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Abstract

The present review summarizes the prevalence and active clinical problems in obese patients with *Helicobacter pylori* (*H. pylori*) infection, as well as the outcomes after bariatric surgery in this patient population. The involvement of *H. pylori* in the pathophysiology of obesity is still debated. It may be that the infection is protective against obesity, because of the gastritis-induced decrease in production and secretion of the orexigenic hormone ghrelin. However, recent epidemiological studies have failed to show an association between *H. pylori* infection and reduced body mass index. *H. pylori* infection might represent a limiting factor in the access to bariatric bypass surgery, even if high-quality evidence indicating the advantages of preoperative *H. pylori* screening and eradication is lacking. The clinical management of infection is complicated by the lower eradication rates with standard therapeutic regimens reported in obese patients than in the normal-weight population. Prospective clinical studies to ameliorate both *H. pylori* eradication rates and control the clinical outcomes of *H. pylori* infection after different bariatric procedures are warranted.

Key words: *Helicobacter pylori*; Obesity; Bariatric surgery; Ghrelin; Antibiotic resistance

Core tip: This review deals with the active clinical problems related to *Helicobacter pylori* (*H. pylori*) infection in obese patients. Even if still controversial, the infection might represent a confounding and limiting factor in bariatric surgery, due to the high incidence of postoperative foregut symptoms and/or lesions in non-eradicated patients. The controversies on preoperative *H. pylori* screening are highlighted, as well as those related to its clinical management, which is complicated by the low eradication rates in obesity. Finally, a revision of studies on the possible correlation between *H. pylori* and body mass index and its possible protective role in development of obesity is included.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is one of the most common human infections and it is estimated that more than half

of the world population is infected[1]. *H. pylori* is an ancient colonizer of the human stomach and represents the main etiological factor in the development of gastritis, peptic ulcer and gastric malignant lesions. The infection is still highly prevalent in developing countries but it is disappearing in the developed world (Table 1; modified from[2]). Indeed, epidemiological studies show that *H. pylori* infection still occurs more frequently in socioeconomically deprived populations living in crowded places with poor hygienic conditions, and conversely, has a lower frequency in people of high socioeconomic status[3]. The influence of these factors on *H. pylori* infection in obesity remains to be ascertained. A study on obese patients who were candidates for bariatric surgery[4] highlighted race as a risk factor for *H. pylori* infection, in that African-Americans and Hispanics had a higher probability than Caucasians of being infected, with significant odds ratios of 4.05 and 2.6, respectively. Interestingly, these two ethnic groups, at least in the United States, have the highest obesity rates[5].

Table 1 Prevalence of *Helicobacter pylori* infection in the general population worldwide

	Country	Prevalence (%)
North America	United States and Canada	30.0
	Canada	23.1
South America	Mexico	70-90
	Chile	70-90
	Brazil	82.0
Europe	Poland	84.2
	Albania	70.7
	Estonia	69.0
	Germany	48.8
	Czech Republic	42.1
	Iceland	36.0
	Switzerland	11.9
	Sweden	11.0
Middle East	Egypt	90.0
	Turkey	80.0
Asia	Bangladesh	> 90.0
	India	88.0
	Japan	55.4
	Taiwan	45.1
Africa	Ethiopia	> 95.0
	Nigeria	70-90
Australia		15.4

PREVALENCE OF *H. PYLORI* INFECTION IN OBESE PATIENTS

The prevalence of *H. pylori* infection in morbidly obese patients is still controversial. Candidates for bariatric surgery have a preoperative prevalence of *H. pylori* ranging from 8.7% in a German cohort[6] to 85.5% in a Saudi cohort[7], with other series showing intermediate values. Overall, available studies report a lower prevalence of *H. pylori* infection in obese patients than in the general population (Table 2). Nevertheless, the few studies that have compared simultaneously the prevalence between lean and obese patients are in disagreement, with half[3,4,8] showing a higher *H. pylori* prevalence in obese patients and the other half[18,23,27] an opposite trend. Discrepancies might be related to both small sample size and variability in diagnostic testing. Different methods are used to diagnose *H. pylori* infection. Serology was largely used in these studies but it has a low diagnostic accuracy of only 80%-84%, and is only useful to exclude *H. pylori* infection. Indeed, positive serology should be confirmed by a test for active infection, such as stool antigen assay or urea breath test (UBT). The former has a sensitivity of 94% and a specificity of 92%, whereas UBT has a sensitivity of 88%-95% and specificity of 95%-100%. Finally, histology has an excellent sensitivity and specificity, especially when specific immunostaining is used to detect *H. pylori* bacteria, and could be partially replaced by biopsy urease testing in patients who have discontinued treatment with proton pump inhibitors or antibiotics[1].

The majority of studies have used only one method to diagnose *H. pylori* infection. When more than one test was used, single tests were not compared with each other and used alternatively. The only study[14] that compared serology to histology found good accordance between the two methods. Testing for active infection has been rare. UBT and stool antigen assay have been used only in one study[12], without any comparison with histology. Even though antisecretory therapy affects diagnostic accuracy of most *H. pylori* diagnostic tests, patients with ongoing antisecretory treatment have been excluded only in one study[22].

H. pylori infection plays a special role in obesity for two main reasons: (1) its possible relationship with body mass index (BMI); and (2) it is a negative factor in limiting access to bariatric surgery.

H. PYLORI INFECTION AND PATHOPHYSIOLOGY OF OBESITY

Both environmental and host agents are involved in the pathophysiology of obesity, including diet, physical inactivity, and drug use, but also genetics and neurophysiological factors. However, an established factor in the development of obesity is dysregulation of the mechanisms that control food intake; mainly under the control of the hormones leptin and ghrelin that are secreted by the gastric mucosa. Leptin primarily suppresses food intake and therefore induces weight loss. Ghrelin increases abruptly before the onset of a meal and decreases rapidly after eating; it has orexigenic effects and inhibits the secretion of leptin. The relationship between ghrelin and *H. pylori* infection in obesity has received much attention in recent years because *H. pylori* gastric mucosal colonization may impair gastric ghrelin production, with subsequent reduction of hunger[28]. Consequently, it has been hypothesized that *H. pylori* infection might have a protective role against obesity.

Several studies[29-32] have investigated the relationship between *H. pylori*-related gastritis and ghrelin, by comparing *H. pylori*-positive with *H. pylori*-negative patients, or

Table 2 Prevalence of *Helicobacter pylori* infection in obese and normal weight patients worldwide

	n	% *H. pylori* + obese (*n*)	% *H. pylori* + control (*n*)	Country	*H. pylori* detection	Ref.
North America	202	6.9 (101)	4.9 (101)	United States	Histology	[8]
	611	23.7	ND		Histology	[9]
	169	30.1	ND		Histology	[10]
	74	24.0	ND		Urease test	[11]
	87	20.0	ND		Urease test	[12]
	58	12.0	ND		Mixed	[13]
	96	11.0	ND		Mixed	[14]
	2684	61.3 (240)	48.2 (2244)		Serology	[4]
	259	22.4	ND		Serology	[15]
South America	232	5.0	ND	Chile	Histology	[16]
	126	53.2	ND	Brazil	Histology	[17]
	83	50.0 (50)	66.6 (33)	Brazil	Histology	[18]
	42	50.0	ND	Brazil	Urease test	[19]
	533	52.5	ND	Chile	Urease test	[20]
	96	37.5	ND	Brazil	Mixed	[21]
Europe	319	39.0	ND	Switzerland	Urease test	[22]
	69	8.7	ND	Germany	Urease test	[6]
	224	23.0 (42)	77.0 (182)	Greece	Serology + histology	[23]
Middle East	656	7.3	ND	Kuwait	Histology	[24]
	62	85.5	ND	Saudi Arabia	Histology	[7]
	214	57.2 (103)	27.0 (111)	Turkey	Serology	[3]
Asia	152	41.4	ND	Taiwan	Histology	[25]
	156	39.7	ND		Histology	[26]
	1097	43.7 (414)	60.0 (683)		Serology	[27]

ND: Not defined; *H. pylori*: *Helicobacter pylori*.

patients before and after eradication treatment, analyzing circulating ghrelin levels, the number of ghrelin immunoreactive cells per cubic millimeter in gastric mucosa[33-35] and ghrelin mRNA expression[33,36,37]. The results of these studies have been conflicting, however, a recent systematic review[38] concluded that circulating ghrelin levels are lower in the presence of *H. pylori* infection. Moreover, studies dealing with morbidly obese patients who are candidates for bariatric surgery have shown discordant results with regard to the relationship between *H. pylori* and ghrelin. Obese patients have been reported to have a reduced[26] or increased[18] number of immunoreactive ghrelin cells, even if their number was lower in *H. pylori*-positive than -negative patients.

A possible explanation of these conflicting results can be ascribed to *H. pylori*-related gastritis patterns. The outcome of *H. pylori* infection differs according to gastritis extension, which generally starts with antritis subsequently progressing to pan-gastritis, which may be associated with atrophy. In the presence of atrophy, secretory activity decreases, which probably involves ghrelin production[39]. Upper gastrointestinal endoscopy with multiple mucosal sampling is required to define the pattern of *H. pylori*-related gastritis, even if an indirect non-invasive serological method is represented by the ratio between pepsinogen (PG) I and Ⅱ; a low PG Ⅰ / Ⅱ ratio being suggestive of the presence of atrophy[40]. By the use of PG Ⅰ / Ⅱ ratio, it has been shown that, within 1 year of *H. pylori* eradication, BMI increased only in the low PG Ⅰ / Ⅱ ratio group[41], suggesting that the sole presence of atrophy has a relevant role in influencing body weight. Indeed BMI, after adjusting for sex and age, was significantly lower in patients with atrophic gastritis than in those without atrophy[42]. Other factors can contribute to explain the controversial results about the relationship between *H. pylori* and ghrelin. First, the different time elapsing from eradication might have influenced the mucosal healing process and then restored normal secretory activity. Second, age might play a role because atrophy is more frequent in elderly patients. Finally, changes in plasma ghrelin concentrations are not strictly associated with gastric mucosal expression and this may be attributed to the presence of ghrelin isoforms with different biological activities[43].

H. PYLORI INFECTION AND BMI

Even if the significant increase in BMI observed after *H. pylori* eradication treatment highlights a possible inverse correlation between *H. pylori* infection and obesity, both in adults[44,45] and children[46], available data are controversial. Indeed, several studies did not find any influence of bacterial eradication on body weight[37,47]. Furthermore, epidemiological studies have failed to show any association between *H. pylori* infection and BMI, with a meta-analysis of 18 observational studies, including 10000 subjects, that reported a slightly higher BMI in *H. pylori*-positive patients[48].

Two theories emerge from these studies. Studies that observed a significant increase in BMI after successful *H. pylori* eradication support a protective role of infection towards obesity, which likely occurs through decreased production and secretion of the orexigenic hormone ghrelin[49]. Studies reporting a higher prevalence of infec-

tion in obese patients disclaim a protective role of *H. pylori* and support the increased incidence and severity of infection observed in obese patients[3]. Obesity can alter innate and adaptive immunity, with immunological impairment related to the grade of obesity, resulting in less maturation of monocytes into macrophages, reduced polymorphonuclear bactericidal capacity, and a significant decrease in NK cell activity[4].

However discrepancies among studies can be ascribed to other factors. As previously mentioned, a misleading factor is the frequent lack of classification of the patients according to their gastritis patterns, especially atrophy[49]. Furthermore, the relationship between *H. pylori* and BMI can be strongly influenced by the dietary change that can occur after eradication. Indeed, *H. pylori* eradication can ameliorate dyspeptic symptoms favoring dietary excess.

H. PYLORI AND BARIATRIC SURGERY

In the field of bariatric surgery, the American Association of Clinical Endocrinologists/The Obesity Society/American Society for Metabolic and Bariatric Surgery guidelines[50] do not provide clear indication about preoperative *H. pylori* screening and management. The document recommends *H. pylori* screening in patients belonging to high-prevalence areas and upper endoscopy in selected cases. Previous European guidelines[51] recommended upper gastrointestinal endoscopy before bariatric surgery in any symptomatic or asymptomatic patient in order to treat any lesions, including *H. pylori* infection, that may cause postoperative complications. The advantages of preoperative *H. pylori* screening and eradication are still controversial, mainly due to a lack of randomized control trials (RCTs). However, different attitudes might be influenced by the differences in health systems and access to upper gastrointestinal endoscopy.

Routine upper endoscopy studies, with concurrent *H. pylori* screening and biopsies to rule out pathological abnormalities (*e.g.*, esophagitis, polyps, hiatal hernia, gastritis, and duodenitis), have reported that abnormalities are present in up to 91% of bariatric candidates[12,16,22], with a higher incidence in patients with concomitant *H. pylori* infection[11,14,52]. Some of these alterations are expected to be cured by tailored bariatric surgery (*i.e.*, hiatal hernia and gastroesophageal reflux disease), even if the main concern is represented by postsurgical gastric malignancy, especially after bariatric procedures with gastric bypass. The majority of obese patients with upper gastrointestinal lesions at the time of routine preoperative endoscopy are asymptomatic[53], with only 20% of the obese patients with pathological findings presenting with upper gastrointestinal symptoms[6]. Also, esophageal dysmotility, frequently observed in these patients, occurs in the absence of symptoms[54]. The lack of visceral sensation in obese patients has been ascribed to alterations in the autonomic nervous system[55,56]. Thus, the decision to perform endoscopy before bariatric surgery on the basis of clinical presentation may be misleading.

Concerning the management of obese patients who are candidates for bariatric surgery, the main clinical issue is represented by *H. pylori* resistance to antibiotic eradication, which could delay access to bariatric surgery. Obese patients showed a significantly lower rate of eradication than controls, at least to the 7-d regimens[57], with BMI being an independent risk factor for eradication failure. Although the mechanisms by which obese patients have a poor eradication rate remain to be elucidated, it seems likely to be due to the following reasons leading to subtherapeutic drug concentrations. First, the physiological changes that occur in obesity, such as possible delayed gastric emptying[58], may lead to a decrease in the rate of drug absorption, regardless of the characteristics of the drug. Second, the volume of distribution of drugs may be altered in obese patients because the increased adipose tissue mass can influence medications with lipophilic properties[59,60]. Clearly, the need of a tailored eradication regimen for obese patients based on body weight arises, but no clinical trials have compared standard therapy versus weight-based regimens. An increase in the eradication treatment efficacy can be obtained by extending the treatment period[61]. A recent trial, aimed to compare 7- and 14-d first-line treatment with clarithromycin-based triple therapy in obese patients, showed that the latter is more effective[62].

EFFECT OF *H. PYLORI* INFECTION ON BARIATRIC SURGERY OUTCOMES

The majority of the studies, focused on standard laparoscopic Roux-en-Y gastric bypass (LRYGB) outcomes, have reported a reduced prevalence of postsurgical lesions after successful *H. pylori* eradication. After LRYGB, *H. pylori* eradicated patients present with a reduced incidence of viscus perforations[13] and of postoperative marginal ulcers[52]. In this latter retrospective study of 560 patients, the incidence of ulcers was 2.4% in patients that were tested and treated for *H. pylori* infection prior to surgery compared to 6.8% in those who did not undergo such screening. However, other authors have reported that, even though marginal ulcer rates following gastric bypass were higher in patients with *H. pylori*, the higher risk persisted even if the pathogen had been eradicated[63]. Indeed, after either LRYGB[64] or laparoscopic sleeve gastrectomy[65,66], postsurgical lesions, mainly concerning gastric ulcers, are attributed to surgical procedures, and not to *H. pylori* infection. Moreover a recent retrospective study did not show any effect of *H. pylori* status, whether preoperatively positive or persistently positive after treatment, on the rates of marginal ulcer or stomal stenosis in patients undergoing LRYGB[67]. So far, high-quality evidence indicating the advantages of preoperative *H. pylori* screening and eradication is lacking and prospective well-designed RCTs are necessary to establish the real clinical outcomes of *H. pylori*-positive and -negative patients after surgery. It should also be considered that the gastric environment for *H. pylori* colonization may dramatically

change after bariatric surgery[50] with possible spontaneous clearance of infection[68].

If the management of *H. pylori* infection in obese patients who are candidates for bariatric surgery is still controversial, there are plausible reasons to attempt eradication in *H. pylori*-positive patients, particularly in those undergoing LRYGB in whom a large part of the stomach is inaccessible to upper endoscopy after surgery. First, eradication should decrease the risk of gastroduodenal peptic lesions in the gastrojejunostomy site after gastric bypass, and thus decrease early as well as later ulcer-related postoperative symptoms and complications, which are higher in *H. pylori*-positive patients[69]. Second, there is evidence of a moderate benefit of *H. pylori* on symptomatic dyspepsia[70,71]. Third, *H. pylori* is a class I carcinogen in the development of gastric cancer with an odds ratio of 2.0-5.9[72]. A recent systematic review[73] on upper gastrointestinal malignancy after bariatric surgery concluded that, even if the incidence were rare, it is advisable to screen patients before surgery because adenocarcinoma, strictly related to *H. pylori* infection, was present in most cases. In addition, the coexistence of *H. pylori* infection with obesity can potentiate the cytokine-mediated crosstalk between inflamed gastric and adipose tissues, augmenting immune responses at both sites, and thereby contributing to a pro-tumorigenic gastric microenvironment[74].

CONCLUSION

Available data on *H. pylori* infection and obesity are still controversial. Current guidelines do not indicate clearly the management of *H. pylori* infection in obese patients who are candidates for bariatric surgery, and the need for *H. pylori* screening and eradication before surgery is still debated. The eradication treatment is often hampered by the low eradication rates obtained with first-line 7-d treatment. Prospective clinical studies aimed to ameliorate both *H. pylori* eradication rates and to evaluate the clinical outcomes of *H. pylori* infection after the different bariatric procedures are warranted.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (6): *Helicobacter pylori*

Helicobacter pylori and pregnancy-related disorders

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Abstract

Helicobacter pylori (*H. pylori*) infection is investigated in gastric diseases even during pregnancy. In particular, this Gram-negative bacterium seems to be associated with hyperemesis gravidarum, a severe form of nausea and vomiting during pregnancy. During the last decade, the relationship among *H. pylori* and several extra-gastric diseases strongly emerged in literature. The correlation among *H. pylori* infection and pregnancy-related disorders was mainly focused on iron deficiency anemia, thrombocytopenia, fetal malformations, miscarriage, pre-eclampsia and fetal growth restriction. *H. pylori* infection may have a role in the pathogenesis of various pregnancy-related disorders through different mechanisms: depletion of micronutrients (iron and vitamin B_{12}) in maternal anemia and fetal neural tube defects; local or systemic induction of pro-inflammatory cytokines release and oxidative stress in gastrointestinal disorders and pre-eclampsia; cross-reaction between specific anti-*H. pylori* antibodies and antigens localized in placental tissue and endothelial cells (pre-eclampsia, fetal growth restriction, miscarriage). Since *H. pylori* infection is most likely acquired before pregnancy, it is widely believed that hormonal and immunological changes occurring during pregnancy could activate latent *H. pylori* with a negative impact not only on maternal health (nutritional deficiency, organ injury, death), but also on the fetus (insufficient growth, malformation, death) and sometime consequences can be observed later in life. Another important issue addressed by investigators was to determine whether it is possible to transmit *H. pylori* infection from mother to child and whether maternal anti-*H. pylori* antibodies could prevent infant's infection. Studies on novel diagnostic and therapeutic methods for *H. pylori* are no less important, since these are particularly sensitive topics in pregnancy conditions. It could be interesting to study the possible correlation between *H. pylori* infection and other pregnancy-related diseases of unknown etiology, such as gestational diabetes mellitus, obstetric cholestasis and spontaneous preterm delivery. Since *H. pylori* infection is treatable, the demonstration of its causative role in pregnancy-related disorders will have important social-economic implications.

Key words: *Helicobacter pylori*; Pregnancy; Hyperemesis gravidarum; Iron deficiency anemia; Pre-eclampsia; Fetal growth restriction; Gastrointestinal disorders

Core tip: *Helicobacter pylori* (*H. pylori*) infection in pregnancy is not only associated with gastrointestinal disorders such as hyperemesis gravidarum, but also with iron deficiency anemia, fetal malformations, miscarriage, pre-eclampsia and fetal growth restriction. These pregnancy related-disorders are potentially life-threatening for both mother and fetus/neonate. Another important issue that has been addressed in literature was the question of whether it is possible to transmit *H. pylori* infection from mother to child and whether maternal anti-*H. pylori* antibodies could prevent infant's infection. Indeed, if *H. pylori* is actually a causal factor, the public health implications would be important since the infection is treatable.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) infection affects approximately one half of the world population and it is more prevalent in developing countries[1,2]. This microorganism colonizes the stomach. Typically, it is acquired during childhood and causes asymptomatic chronic infection[2]. A small portion of *H. pylori* infected subjects develop peptic ulcers and gastric carcinoma, usually during late adulthood[2].

H. pylori pathogenicity depends on several strain-specific factors. Some *H. pylori* strains express specific genes conferring pro-inflammatory, cytotoxic and vacuolating properties which could enhance the *in vivo* pathogenicity[3]. Virulence factors such as urease and flagella are present in all strains and they are pivotal for pathogenesis and colonization[4]. Adhesins, such as Outer inflammatory protein and Sialic acid-binding adhesin, facilitate bacterial attachment to the host epithelium and often induce its inflammatory response[5,6]. *H. pylori*-strains can also express Cytotoxin-associated antigen A (CagA) and Vacuolating cytotoxin A (VacA), the most investigated cytotoxins among *H. pylori* virulence factors. CagA is directly injected into the cytoplasm of epithelial cells, affecting cell morphology, proliferation and apoptosis[7]. *H. pylori* strains carrying CagA have been associated with both duodenal ulcer and gastric cancer[8], and infection with CagA-positive strain is generally associated to higher levels of inflammatory mediators compared to CagA negative strains[3]. VacA is a proteic pore-forming toxin crucial to promote and maintain bacterial colonization[9]. It disrupts cell polarity, promotes epithelial cells apoptosis and inhibits T cell proliferation and effector function[10]. Interestingly, combined seropositivity for both CagA and VacA directly correlates with elevated morbidity[11-13].

During the past decades, several reports indicated a correlation between *H. pylori* infection and various extragastric disorders[14]. Such manifestations include ischemic heart disease, diabetes mellitus, idiopathic thrombocytopenia, urticaria, and sideropenic anemia[14]. Lanciers *et al*[15] (1999) found a significantly increased incidence of pregnant subjects with high *H. pylori* IgM (marker for recently acquired infection) compared to non pregnant women. These Authors suggested that pregnancy itself may increase the susceptibility to *H. pylori* infection[15]. This is probably due to the fact that there are immunologic adaptations in pregnancy to ensure maternal tolerance towards the semi-allogeneic fetus. In general, pregnancy is characterized by a decreased cell-mediated cytotoxic immune response with preservation of humoral and innate immunity[16].

Nowadays, no follow-up study was conducted to describe the complete immune response against *H. pylori* infection during pregnancy. Most studies on the correlation between *H. pylori* infection and pregnancy-related disorders were cross-sectional investigations where *H. pylori* positivity was detected during pregnancy or soon after delivery. *H. pylori* infection was tested before conception only in one prospective study, where early pregnancy loss was associated with maternal *H. pylori* CagA-strains seropositivity before intra-cytoplasmic sperm injection[17]. Indeed, it is not possible to definitely conclude whether pregnancy-related complications are correlated to *H. pylori* infection acquired before or during pregnancy.

The prevalence of *H. pylori* infection in pregnant women varies according to geographic area, socioeconomic conditions and method used to detect *H. pylori* infection. For example, the prevalence of *H. pylori* infection among pregnant women is about 20%-30% in most European countries[18-20], Japan[21] and Australia[22], while it is 50%-70% in Turkey[23,24], Mexico and in Texas, United States[25,26], more than 80% in Egypt[27] and Gambia[28]. Furthermore, inadequate sanitation practices, low social class and crowded or high-density living conditions seem to be related to a higher prevalence of *H. pylori* infection. These observations suggest that poor hygiene and crowded conditions may facilitate transmission of infection among family members and they are consistent with data on intra-familial and institutional clustering of *H. pylori*[29,30].

The first investigations on pregnancy focused their attention mainly on the relationship between hyperemesis gravidarum and *H. pylori* infection. Next, researchers turned their attention to other pregnancy-related disorders, such as iron deficiency anemia, thrombocytopenia, fetal growth defects and malformations, miscarriage and, more recently, to pre-eclampsia. Another important issue that has been addressed was the question of whether it is possible to transmit *H. pylori* infection from mother to child and whether maternal anti-*H. pylori* antibodies could prevent infant's infection. Finally, investigations on diagnostic and therapeutic methods for *H. pylori* are no less important, since they are particularly sensitive topics in pregnancy-related conditions. Herein, we reviewed the up-to-date literature about *H. pylori* and pregnancy.

GASTROINTESTINAL DISORDERS IN PREGNANCY

Mild to moderate dyspepsia is commonly associated with nausea and vomiting and complicates about 50% of all pregnancies and it diminishes women's life quality and social functions during early pregnancy[31]. In most women, these symptoms resolve by fluid and vitamin supplementation as well as dietary modification. About 0.3%-2% of pregnant women suffer from Hyperemesis Gravidarum (HG) characterized by severe and protracted vomiting that often results in dehydration, electrolyte imbalance, ketonemia, ketonuria, and weight loss[31-34].

Dehydration and acid base disturbances may lead to renal and hepatic injury[35]. Patients who manifest continuous weight loss and electrolyte disturbances may be at risk for growth restriction, fetal anomalies and decreased neonatal birth weight[36].

The onset of gastrointestinal symptoms is always during the first trimester, but HG may persist throughout gestation. The etiology of HG, which still remains unknown, seems to be multifactorial and may be the final result of various unrelated conditions. Indeed, treatment is performed on a symptomatic basis[35]. In particular, psychological causes, gastrointestinal tract dysfunctions, endocrine factors (*i.e.*, elevated human chorionic gonadotropin and estrogen), genetic incompatibility, immunological factors and nutritional deficiencies have been considered part of the pathologic mechanism underlying HG. However, no single theory seems to provide an adequate explanation for HG[33,35].

Significant positive association between HG and *H. pylori* infection has been demonstrated by several case-control studies[37-42], and in a systematic review of 14 case-control studies, Golberg *et al*[32] (2007) found higher prevalence of HG in *H. pylori*-infected pregnant women than uninfected ones (pooled OR = 4.45; 95%CI: 2.31-8.54). In contrast, most of the studies aimed to determine the link between *H. pylori* and dyspepsia failed to show a significant correlation between the clinical symptoms of the disease and *H. pylori* infection[43,44]. Only two studies investigated the relationship between CagA-positive *H. pylori* strains and gastrointestinal problems in pregnancy. Noyan *et al*[45] (2004) found a significant association between CagA-seropositivity and dyspepsia in pregnancy, though *H. pylori*-seroprevalence resulted slightly but not significantly higher in pregnant women with dyspeptic complaints (74.6%) compared to the controls (63.8%). Xia *et al*[46] (2004) demonstrated that the infection rates of both *H. pylori* and CagA-positive strains are significantly higher in HG patients (88.9% and 78.1%, respectively) than in asymptomatic pregnant women (45.0% and 31.3%, respectively) ($P < 0.01$ for both).

Despite a high seropositive rate in pregnant women with severe gastrointestinal symptoms during early pregnancy, no correlation was found between seropositivity and clinical symptoms or their duration[47,48]. Shirin and colleagues (2004) reported an association between *H. pylori* and mild vomiting during early pregnancy but not with gastrointestinal symptoms later in pregnancy[49]. Studies performed on endoscopic biopsies of gastric mucosa demonstrated that the severity of gastrointestinal symptoms in early pregnancy may be associated with the density of *H. pylori* in the gastric epithelium[50]. Additionally, two case reports showed that *H. pylori* eradication treatment reduces the severity of HG[51,52].

In contrast, several studies found no relationship between HG and *H. pylori*[24,53-55]. These contradictory findings are probably due to the fact that a universally accepted HG definition does not exist, thus indicating a high heterogeneity of the study population.

It has been proposed that a reduction of gastric acid production during early pregnancy as a result of increased accumulation of woman's body fluid, steroid hormone changes, and immunologic tolerance could lead the activation of latent *H. pylori* infection, which can exacerbate nausea and vomiting symptoms[42].

IRON DEFICIENCY ANEMIA

Iron deficiency is the most common nutritional deficiency in the world and results in impairment of immune, cognitive and reproductive functions, as well as decreased work performance[56]. Iron deficiency anemia (IDA) affects more than a billion people worldwide and contributes to up to 40 percent of maternal deaths in the developing countries[57]. In a typical singleton pregnancy, the average daily demand for iron is approximately 4.4 mg. A supplementation is needed when diet alone cannot supply this amount of iron, but despite iron supplementation, many women continue to remain anemic[58].

Muhsen *et al*[59] (2013) recommended the investigation of *H. pylori* infection as a potential factor that might play a role in the occurrence of anemia in children and pregnant women. Furthermore, eradication of *H. pylori* infection has been recommended for patients with unexplained IDA[60,61]. These recommendations are based on several studies that found a relationship between *H. pylori* and IDA. In a systematic review and meta-analysis of 12 case reports and series, 19 observational epidemiologic studies and six interventional trials, Muhsen and Cohen (2008) found higher prevalence of IDA in *H. pylori*-infected subjects than uninfected ones[62]. Several IDA mechanisms have been hypothesized in *H. pylori* infection, some of which are decreased mucosal iron absorption capacity due to low gastric pH, reduction of stomach vitamin C levels, bacterium-host competition for dietary iron supply, lactoferrin mediated iron sequestration by gastric *H. pylori*, increased hepatocytes hepcidin release in response to IL-6 production associated with *H. pylori* gastritis[56,62-67].

In pregnant women, *H. pylori* infection has been found to be associated with IDA[19,68-70]. Weyermann *et al*[19] (2005) found lower haemoglobin (Hb) levels at the beginning of pregnancy in *H. pylori* infected mothers *vs* noninfected (-0.25 g/dL; 95%CI: -0.49--0.003) and a more unfavourable change in Hb level during course of pregnancy (-0.14 g/dL; 95%CI: -0.38-0.10). In a cross-sectional study, out of 117 pregnant women, 27 had anemia and all of the anemic patients were shown to be *H. pylori* infected, and with a high chance of fetal growth restriction[68]. In a small prospective study aimed to confirm the association between *H. pylori* infection and HG, it was found that infected pregnant women with HG have higher prevalence of IDA compared to symptomatic uninfected patients[70]. In a small randomized double-blind placebo controlled trial, high prevalence of *H. pylori* infection was seen in pregnant women suffering from IDA and eradication of

the infection by triple drug therapy during third trimester enhanced the response to oral iron folic acid supplementation[69].

PRE-ECLAMPSIA

Pre-eclampsia (PE) is a pregnancy-related syndrome characterized by new onset hypertension and proteinuria after 20 wk of gestation in a previously normotensive woman. PE affects about 2%-8% of all pregnancies and remains one of the main causes of either maternal or fetal mortality and morbidity worldwide[71]. Despite PE has been object of intense investigation, its etio-pathogenetic mechanisms are still poorly understood. This difficulty is certainly due to the fact that PE is a syndrome where similar symptoms could origin from different pathogenic pathways. PE is characterized by a generalized vascular dysfunction and an excessive maternal inflammatory response. Furthermore, it is possible to recognize two different forms of PE: "placental PE", characterized by abnormal placentation and feto-placental compromise, and "maternal PE", where etio-pathogenic mechanisms do not directly involve placenta and the fetus but they are of exclusive maternal origin[72,73].

Several evidences suggest that subclinical infections could play a role in the onset of PE[74,75]. The association between *H. pylori* seropositivity and PE was found for the first time by our group[76]. We showed that *H. pylori* seropositivity frequency is higher in mothers with PE (51.1%) compared with women with uneventful pregnancy (31.9%) (OR = 2.67; 95%CI: 1.08-6.57; $P = 0.033$)[76]. Afterwards, other two case-control studies reported a significantly higher *H. pylori* seropositivity rate in PE patients compared to controls[77,78]. UstUn *et al*[77] (2010) reported a significantly higher positivity for IgA anti-*H. pylori* in patients with PE compared with controls ($P = 0.034$), and Aksoy and colleagues found a *H. pylori* seropositivity rate of 81% in the pre-eclampsia group, and of 60% in normal pregnant women (OR = 2.86; 95%CI: 1.05-7.82; $P = 0.036$)[78]. We also showed a strong association between the onset of PE and CagA-positive *H. pylori* strains infection, which are more virulent and therefore more likely to elicit the generalized inflammation and the subsequent vascular damage typical of PE[76]. Recently, we found that CagA/VacA dual seropositivity is specifically associated with PE and, in particular, with "placental PE"[79]. Interestingly, Franceschi *et al*[80] (2012) demonstrated that antibodies against the *H. pylori* virulence factor CagA cross-react *in vitro* with placental tissue reducing its invasiveness ability and it is well known that these antibodies recognize antigens localized on the surface of endothelial cells[81]. Therefore infection with CagA-positive strains could contribute not only to the exacerbated maternal inflammatory response leading to all forms of PE but also to the abnormal placentation typical of "placental PE".

H. pylori could be involved in the pathogenesis of PE mainly by inducing inflammation and oxidative stress and consequently generalized endothelial dysfunction. In fact, it was observed that *H. pylori* seropositive PE subjects are characterized by a more severe inflammatory status compared to the inflammatory response characterizing normal pregnancy, since pre-eclamptic women showed higher levels of C-reactive protein, tumour necrosis factor (TNF)-alpha and maternal leukocytes counts[77,79]. Interestingly, pre-eclamptic patients, had higher *H. pylori* seropositivity rate and serum malondialdehyde levels, a common marker of lipid peroxidation, compared with healthy pregnant. Furthermore, the subgroup of seropositive PE mothers had higher serum levels of total cholesterol and low-density lipoprotein (LDL)-C compared to seronegative PE women. The Authors hypothesized that *H. pylori* infection may be a contributory factor in atherosclerosis in PE cases later in life[78]. Prospective cohort studies are required to confirm this hypothesis. However, such studies would be difficult to be conducted, since large cohorts of pregnant women would be needed to detect significance and prospective studies are limited in their ability to evaluate uncommon outcomes such as PE.

FETAL GROWTH RESTRICTION

Fetal growth restriction (FGR) is defined as failure of the fetus to achieve its genetically determined growth potential[82,83]. FGR may be due to either feto-placental or maternal causes and 3%-10% of infants suffer from growth restriction. Feto-placental causes include infections and other placental pathologies. Known maternal causes of FGR include vascular disorders (chronic hypertension, pre-eclampsia or diabetes with vasculopathy), poor maternal weight gain, smoking, alcohol, cocaine, advanced maternal age and previous poor pregnancy outcome[84].

Eslick *et al*[22] (2002) observed for the first time an association between *H. pylori* infection and low birth weight, in particular they showed that intrauterine growth restriction was more common in *H. pylori* seropositive women (13.5%) than in seronegative mothers (6.0%) (OR = 2.41; 95%CI: 1.14-5.08; $P = 0.018$). Furthermore, it has been reported that *H. pylori* infected mice showed a decrease in implantation rates, and their offspring were of low birth-weight[85]. However, in another experimental mice model study these results were not confirmed[86].

H. pylori may be linked with an increase in symptoms including dyspepsia, nausea or vomiting[42,52], because of underlying undiagnosed peptic ulcer disease, which in turn may affect maternal gastric absorption and therefore impair fetal growth. Also maternal anemia associated to *H. pylori* infection may lead to FGR. In fact, Mulayim *et al*[68] (2008) observed that pregnant women with *H. pylori* infection delivered neonates with a significantly lower birth-weight compared to mothers without the infection. However, in this study FGR could be due to maternal anemia since all anemic pregnant women were in the *H. pylori* positive group. As previously underlined,

it was recently demonstrated that anti-CagA antibodies cross-react *in vitro* with placental tissue reducing its invasiveness ability[80] and the consequent abnormal placentation could lead to FGR. However, in our study on *H. pylori* virulence factors we demonstrated a strong association between *H. pylori* infection and FGR in pre-eclamptic pregnancies, while there was no association between *H. pylori* and idiopathic FGR[79].

OTHER PREGNANCY-RELATED DISORDERS

H. pylori infection has been linked to other few disease states in pregnancy but there are still small amount of data supporting these premises.

Miscarriage

Miscarriage or spontaneous abortion, occurring in 15% of pregnancies, is defined as an unintended termination of pregnancy resulting in fetal death prior to 23 wk of gestation[87]. Among non-chromosomal causes of fetal loss, infections have a minor relevance compared to other etiologic factors. However Rossi *et al*[85] (2004) observed a higher number of fetal resorption in *H. pylori* infected pregnant mice compared to non-infected controls. Hajishafiha *et al*[17] found an association between *H. pylori* CagA-strains maternal infection and early pregnancy loss in patients undergoing intra-cytoplasmic sperm injection. Recently we found a significantly higher percentage of *H. pylori* seropositive women among primigravidae with a miscarriage compared to controls, while the presence of maternal serum antibodies against *H. pylori* did not appear to be associated with recurrent miscarriage[20]. These findings suggest a relationship between *H. pylori* infection and implantation/placentation failure, possibly due to a cross-reaction between antibodies against *H. pylori* and placental tissue[80].

Neural tube defects

Several studies reported that serum/plasma vitamin B12 and folate levels are lower in subjects with *H. pylori* infection compared to uninfected persons[88,89]. Moreover, several investigations indicated that vitamin B_{12} and folate levels improve after *H. pylori* eradication[90,91]. Two case-control studies in a Mexican-American and in Iranian population reported that *H. pylori* could play a role in neural tube defect (NTD) causation by reducing folate and vitamin B_{12} concentrations. They showed that *H. pylori* seropositivity in pregnant women can increase the risk of occurrence of NTDs in newborns, since seropositivity was more frequent among mothers of newborns with NTDs than controls[25,92]. However in both studies the differences were not significant.

Thrombocytopenia

Thrombocytopenia, although often innocuous[93], could have dangerous complications during pregnancy. Pregnancies affected by extremely low platelets, often with immune (idiopathic) thrombocytopenic purpura (ITP), require frequent careful monitoring during prenatal visits, especially once entering the third trimester in preparation for delivery[94]. Furthermore, a recent retrospective study showed that ITP was an independent risk factor for both perinatal mortality and preterm delivery[95].

Association between *H. pylori* and thrombocytopenia has been demonstrated in a non-pregnant population[96-98]. The etiology of thrombocytopenia may be due to cross-molecular mimicry between specific *H. pylori* protein (CagA) and platelet antigens[99], however no relationship was found between *H. pylori* infection and platelet count during pregnancy[68,79,100,101].

It would be interesting to confirm the above mentioned findings and to investigate the possible correlation among *H. pylori* infection and other pregnancy-related diseases of unknown etiology, such as gestational diabetes mellitus, obstetric cholestasis and spontaneous preterm delivery. In fact, *H. pylori* infection seems to be associated to diabetes mellitus[102] and hepatobiliary diseases in the general population[103] and it is well known that bacterial infections increase the risk of spontaneous preterm delivery[104].

MOTHER-TO-CHILD TRANSMISSION

Children of *H. pylori* infected mothers seem to have a higher risk of acquiring *H. pylori*[27,105]. However, experimental animal models suggested that vertical infection during the prenatal period or delivery procedure is unlikely to be route of mother-to-child transmission of the infection. It is possible that *H. pylori* is acquired through breast-feeding, contaminated saliva and fecal-oral transmission during co-habitation[21,86,106]. Indeed, for the general population, the most common way of transmission is from person to person by either oral-oral route (through vomitus or possibly saliva) or fecal-oral route. The person-to-person way of transmission is supported by the higher incidence of infection among institutionalized children and adults and the clustering of *H. pylori* infection within families. Moreover, detection of *H. pylori* DNA in vomitus, saliva, dental plaque, gastric juice, and feces further supports this concept. Waterborne transmission, probably due to fecal contamination, may be an important source of infection, especially in those world's areas in which untreated water is common[29,30].

Furthermore, in our previous study on pre-eclampsia and *H. pylori* we indirectly demonstrated the absence of vertical transmission in humans, since we never found the presence of *H. pylori* DNA in placentae of *H. pylori* positive patients[76].

It is widely established that specific anti-*H. pylori* IgG antibodies are transplacentally transferred from mothers to fetuses[18] and a close correlation between maternal and cord specific IgG levels was demonstrated[21,28]. These passively acquired antibodies decline over the first 3-4 mo of life[18,28]. Some researchers have suggested that maternal IgG may protect against *H. pylori* colo-

nization[18,107] and this is supported by work in murine models[108]. Other investigators found no evidence of a protective role for passively acquired maternal antibodies in infants at high risk of early *H. pylori* colonization[28].

It was also suggested that IgA antibodies in maternal milk confer passive protection against early human *H. pylori* colonization[109-111]. However, in a previous study the relationship between breastfeeding and *H. pylori* was investigated in 946 preschool children and their mothers with C-urea breath test. *H. pylori* prevalence was higher in breastfed children compared with children who were never breastfed. The Authors concluded that breastfeeding was not protective against *H. pylori*[112].

H. PYLORI INFECTION DIAGNOSIS DURING PREGNANCY

The current diagnostic methods include invasive and non-invasive tests. Invasive tests involve an upper gastrointestinal endoscopy with gastric mucosal biopsy and rapid urease activity detection, histology, microbiological culture, or polymerase chain reaction assays. Although mucosal biopsy and histopathologic examination of specimens for the presence of *H. pylori* and/or gastritis is considered the gold standard for the diagnosis of *H. pylori* infection, invasive tests are not well tolerated by patients and may be a source of ethical problems. Gastroscopy can be performed in pregnant patients, but only when it is strictly necessary[113].

The non invasive methods are more widely accepted in the prenatal period and include serum antibody detection, carbon-labeled urea breath tests, and stool antigen detection.

Serologic and stool antigen tests are the first choice for *H. pylori* infection diagnosis in pregnancy, since they are easy to perform and low-cost non invasive diagnostic tests. Serologic tests are usually based on the detection of specific anti-*H. pylori* IgG antibodies in the patients' sera by immuno-enzymatic assay. Measurement of IgG antibodies against *H. pylori* reveals an immune response that could represent either a current infection or a previous exposure, since IgG antibodies disappear only several months after eradication of the microorganism[114].

The stool antigen test is an enzymatic immunoassay that detects the active presence of *H. pylori* antigen in human feces. Stool antigen test is preferred to determine the *H. pylori* status after eradication[115].

Urea breath tests are not commonly used during pregnancy, despite they are reliable and noninvasive diagnostic test. In fact, it is demonstrated that ^{13}C-urea breath test, using the stable isotope ^{13}C as tracer, is not radioactive and safe also in children and pregnancy. Therefore, it could be used as a valuable non-invasive semi-quantitative diagnostic tool for the assessment of gastric bacterial *H. pylori* infection. The urea breath test is recommended for test-and-treat strategies and suitable for control after eradication therapy and in epidemiological or pharmacological studies[116]. Despite the excellent sensitivity and specificity of these tests, they are expensive and require specific instrumentation and specialized staff.

Furthermore, it was stated that ionizing radiation dose involved in ^{14}C-urea breath test is extremely low, much lower than the radiation dose adsorbed from natural sources, a thousand times lower than the amount of fetal radiation considered to be teratogenic, therefore in the event of inadvertent exposure during pregnancy, the pregnant women should be reassured[117].

H. PYLORI INFECTION TREATMENT DURING PREGNANCY

There are multiple options for *H. pylori* infection treatment. The association of a proton-pump inhibitor and two antibiotics for 1 or 2 wk gives the best eradication rates in non pregnant subjects. Currently, there are no guidelines to treat *H. pylori* infection during pregnancy and the optimal therapy in pregnancy remains uncertain[118]. Hayakawa *et al*[119] treated four women with hyperemesis gravidarum by a combination of penicillin and erythromycin, leading to alleviation of symptoms thus demonstrating the possible effectiveness of this specific *H. pylori* treatment. This hypothesis is supported in four additional case reports that showed similar symptom relief after antibiotic treatment[47,51,52,120].

Several investigators have evaluated the safety of individual drugs, including proton pump inhibitors used in the anti *H. pylori* drug therapy in pregnant women. A recent meta-analysis reported that the use of proton pump inhibitors during first-trimester does not seem to be associated with increased risk of spontaneous abortion, preterm delivery or major congenital birth defects[121]. Nevertheless, some experts recommend that *H. pylori* eradication should be deferred until after pregnancy and lactation[122].

It must be considered that treatment of *H. pylori* infection has a low successful rate, with 35%-85% of infections being cleared, reaching the lowest values in some European countries[123]. The gradual but steady occurrence of antibiotic-resistant strains represents a major obstacle in the treatment of *H. pylori* infection. Pharmacogenomics-based approaches seem to increase the cure rates, but re-infection also remains problematic. In fact, it is well known that eradication of *H. pylori* infections with antimicrobial agents in adults does not induce immunity against re-infection. In general, low annual recurrence rates were observed in developed countries (up to 2% for both adults and children), but high recurrence rates (> 10%) were observed in developing countries[124]. There is no clear evidence that pregnancy predisposes to *de novo H. pylori* infection.

In view of these evidences, new approaches need to be considered for treatment of this disease, such as design of effective vaccines. Especially in case of pregnancy related diseases, it would be preferable to prevent *H. pylori* infection consequences, thus avoiding phar-

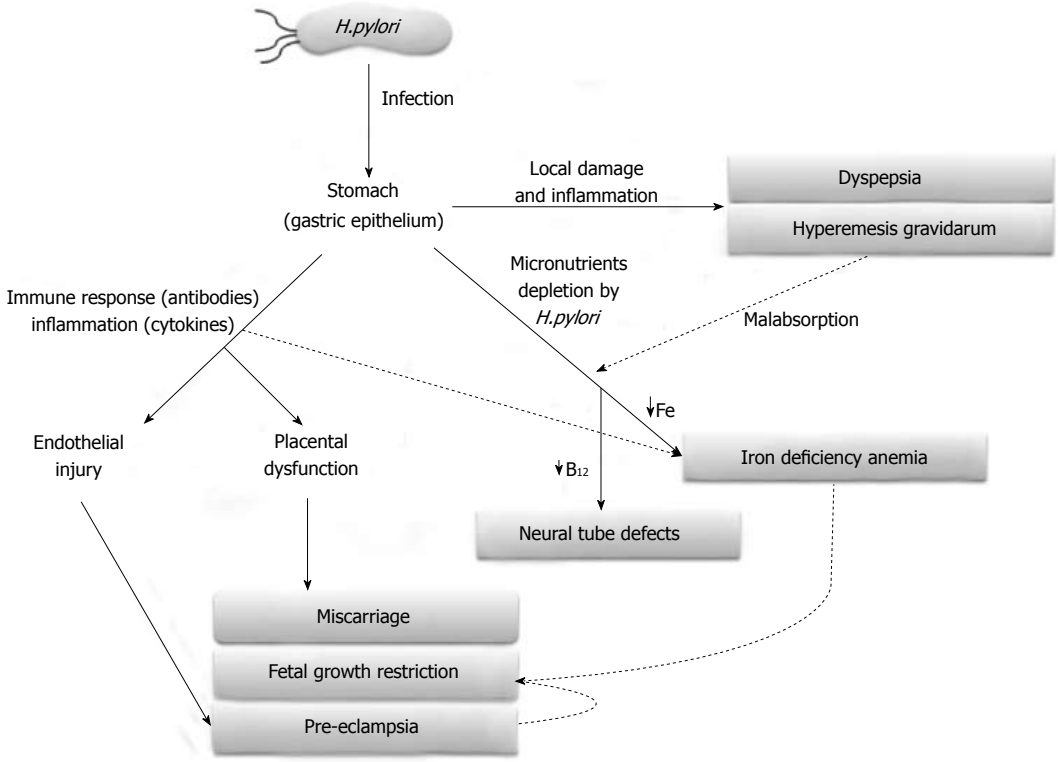

Figure 1 *Helicobacter pylori* infection correlation with pregnancy-related disorders. *Helicobacter pylori* (*H. pylori*) infection can cause local damage and inflammation, leading to gastrointestinal disorders such as dyspepsia in pregnancy and hyperemesis gravidarum. *H. pylori* sequesters essential micronutrients from the host organism. In particular, iron depletion may lead to iron deficiency anemia (IDA), while reduction of vitamin B_{12} and folate may result in fetal neural tube defects. Lack of these micronutrients may also be favored by gastric malabsorption in case of the above mentioned gastrointestinal problems. Furthermore, IDA could indirectly be the consequence of local and systemic inflammation induced by *H. pylori* infection. Finally, the immune and inflammatory responses caused by this infection lead to endothelial and placental injury, through the cross-reaction of anti-*H. pylori* and tissue antigens and through the production of pro-inflammatory cytokines. Placental dysfunction characterizes important diseases of pregnancy, such as miscarriage, fetal growth restriction (FGR) and pre-eclampsia that it is also characterized by endothelial damage and it is often associated with FGR. Furthermore, IDA could be a risk factor for FGR.

macologic therapies during pregnancy. Recently, several clinical trials and animal studies have been focused on generating *H. pylori* recombinant vaccines useful to eradicate and protect against the infection; however a safe and effective *H. pylori* vaccine has not yet been developed for use in humans[123].

Therefore, if *H. pylori* infection will be confirmed as an important risk factor for pregnancy complications, we suggest the conventional *H. pylori* eradication, namely triple therapy, should ideally be obtained several months before conception in order to reach seronegativity. This approach would avoid cross-reaction between anti-*H. pylori* antibodies and host tissue antigens, waiting for the discovery of novel effective vaccines.

CONCLUSION

H. pylori infection was investigated not only in association with gastrointestinal manifestations during pregnancy but also with other severe pregnancy-related disorders. *H. pylori* infection may have a role in the pathogenesis of these disorders through different mechanisms: depletion of micronutrients (iron and vitamin B_{12}) in the case of maternal anemia and fetal neural tube defects; local and systemic induction of pro-inflammatory cytokines release and oxidative stress in gastrointestinal disorders and pre-eclampsia; cross-reaction between specific anti-*H. pylori* antibodies and antigens localized in placental and endothelial cells (pre-eclampsia, fetal growth restriction, miscarriage) (Figure 1). Since *H. pylori* infection is most likely acquired before pregnancy, it is believed that hormonal and immunological changes occurring during pregnancy can activate latent *H. pylori* infection and this could have an impact not only on the mother health (nutritional deficiency, organ injury, death), but also on her child (insufficient growth, malformation, death) and sometime consequences can be observed later in life.

H. pylori mother to child transmission does not appear to occur during pregnancy or delivery. Furthermore, it was demonstrated that specific antibodies against this microorganism are transferred to the fetus/infant both transplacentally and by means of maternal milk. However, it is not clear whether maternal antibodies are able to protect the children against *H. pylori* colonization.

Currently, clinicians choose a non-invasive diagnostic method for *H. pylori* infection and prefer to treat the infection out of pregnancy. If *H. pylori* will be confirmed as causal and/or contributing factor of major pregnan-

cy-related disorders, it will have important positive implications for the public health system since the infection is treatable. It is likely that pre-pregnancy diagnosis and preventive *H. pylori* eradication would reduce the incidence of some of these complications. More data are needed to understand if screening for *H. pylori* infection could be effective in preventing pregnancy disorders. The design of an effective vaccine will be even more useful in order to avoid drug resistance and re-infection problems.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (6): *Helicobacter pylori*

Eradication of *Helicobacter pylori* infection: Which regimen first?

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Abstract

Helicobacter pylori (*H. pylori*) is a well-known human pathogen that plays an essential role in the pathogenesis of chronic gastritis, peptic ulcer disease, and gastric malignancies. Although *H. pylori* is susceptible to several antimicrobials, this infection has proven challenging to cure because of the increasing prevalence of bacterial strains that are resistant to the most commonly used antimicrobials, particularly clarithromycin. An effective (*i.e.*, > 90%) first-line therapy is mandatory for avoiding supplementary treatments and testing, and more importantly for preventing the development of secondary resistance. This study reviews the recent literature on first-line therapies for *H. pylori*. The eradication rates following standard triple therapy (a proton pump inhibitor plus amoxicillin and clarithromycin) for *H. pylori* infection are declining worldwide. Several first-line strategies have been proposed to increase the eradication rate, including extending the treatment duration to 14 d, the use of a four-drug regimen (bismuth-containing quadruple, sequential, and concomitant treatments), and the use of novel antibiotics, such as fluoroquinolones. However, the efficacy of these regimens is controversial. A first-line eradication regimen should be based on what works best in a defined geographical area and must take into account the prevalence of antimicrobial resistance in that region.

Key words: *Helicobacter pylori*; Sequential therapy; Hybrid therapy; Concomitant therapy; Clarithromycin; Levofloxacin

Core tip: First-line therapy for *Helicobacter pylori* infection should have an efficacy higher than 90% to prevent the need for additional treatment and the emergence of secondary antimicrobial resistance. The first-line eradication regimen should be based on what works best in a defined geographical area and must take into account the prevalence of antimicrobial resistance in that region. Non-bismuth quadruple (*i.e.*, concomitant) therapy appears to have high efficacy and, in our opinion, is the first choice of treatment for eradicating the infection.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a global human pathogen that plays a key role in the development of prevalent diseases, including peptic ulcer disease and gastric malignancy[1,2]. Therefore, this infection should be cured whenever it is

diagnosed[3].

Seven- to ten-day triple therapy consisting of a proton-pump inhibitor (PPI) plus amoxicillin and clarithromycin is the standard first-line treatment option for *H. pylori* eradication since its first acceptance in the international guidelines in 1996[4-6]. The efficacy of this treatment is strongly affected by clarithromycin resistance, and we have been witnessing a progressive decline in the eradication rate over the last decade, both in the United States and Europe, to below the acceptability threshold of 80%[7-11]. In some European countries, the success rates are disappointingly low, with values of only 25%-60%[12,13].

Several strategies have been proposed to increase the eradication rate, including the extension of the treatment duration to 14 d, the use of a four-drug regimen (bismuth-containing quadruple, sequential, and concomitant treatments), and the use of novel antibiotics, such as levofloxacin[14-21]. As with other infectious diseases, the treatment results are best when reliably excellent regimens are used to treat patients infected with organisms that are susceptible to the chosen antimicrobials[22,23]. Pretreatment susceptibility testing can be performed directly (by culture of the organism) or indirectly [by molecular testing of the stools of infected patients or by fluorescent in-situ hybridization using paraffin-embedded gastric biopsy samples] and allows the selection of a regimen that is tailored based on antimicrobial susceptibility[7]. However, in many instances, therapy must be chosen empirically, and in this case, the best approach is to use regimens that have proven to be reliable and to perform well locally[24]. This approach should take advantage of knowledge of resistance patterns obtained from local or regional antimicrobial surveillance programs and/or should be based on local clinical experience with regard to which regimens are effective in a given area.

The present article aims to critically assess, through a systematic review of the literature and pooled-data analysis, the current options for *H. pylori* eradication. To this end, we analyzed and compared recent evidence regarding the efficacy of several therapeutic regimens. The advantages and disadvantages of the proposed anti-*H. pylori* regimens, as well as the existing evidence for their clinical validation and widespread use in routine practice, are provided. This article will focus on first-line treatment; therefore, second-line or rescue therapies will not be discussed.

STANDARD TRIPLE THERAPY

Standard triple therapy consists of a 7-10-d regimen with a PPI [standard dose, twice a day (*bid*)], amoxicillin (1 g, *bid*), and clarithromycin (500 mg, *bid*). Clarithromycin resistance is the major cause of eradication failure for standard triple therapy[25]. Pooled data from 20 studies involving 1975 patients treated with standard triple therapy showed an eradication rate of 88% in clarithromycin-sensitive strains *vs* 18% in clarithromycin-resistant strains[26]. Therefore, the background rate of clarithromycin resistance is critically important, as it negatively impacts the efficacy of standard triple therapy. A systematic review showed that the rate of clarithromycin-resistant strains ranged from 49% (Spain) to 1% (The Netherlands) worldwide[27]. In areas with clarithromycin resistance of < 10% [*i.e.*, The Netherlands, Sweden, Ireland, Germany, Malaysia, and Taiwan (South)], it is still possible to employ a standard triple therapy to achieve a per-protocol (PP) eradication rate > 90%. The obsolescence of standard therapies for high clarithromycin resistance areas is now clearly stated in the 2012 Maastricht Ⅳ/Florence consensus report: a threshold of 20% is used to separate the regions of high/low clarithromycin resistance, with clarithromycin-containing regimens maintaining their role as standard therapies only if local resistance to this agent does not exceed 20%[28]. However, standard triple therapy should be abandoned in areas with clarithromycin resistance ⩾ 20% [*i.e.*, Spain, Turkey, Italy (Central), Alaska, China, Japan, and Cameroon] because the PP eradication rates of standard therapy are often less than 85%, and the intention-to-treat (ITT) eradication rates are usually less than 80%[7,26,29].

The use of probiotics has attracted attention as an alternative approach for increasing eradication rates and decreasing treatment-related side effects. The exact role of probiotics in the eradication of *H. pylori* remains largely unknown. However, evidence for an encouraging increase in the eradication rates achieved with standard triple therapy by including *Saccharomyces boulardii*[30] or *Lactobacillus* spp.[31] supplementation has been provided by recent meta-analytical data.

MODIFIED TRIPLE THERAPY

Based on a large body of published clinical trials, a quinolone-containing triple therapy has proven to be effective as a first-line therapy for *H. pylori* infection. The eradication rates of levofloxacin-containing triple therapy ranged from 72% to 96%[30].

This regimen might be considered in populations with clarithromycin resistance greater than 15%-20% and quinolone resistance less than 10%[32]. However, quinolone-containing triple therapy is not generally recommended as a first-line therapy at the moment due to concerns about the rising prevalence of quinolone-resistant *H. pylori* strains. Furthermore, greater use of quinolones would likely result in the development of more quinolone-resistant pathogens responsible for respiratory and urogenital tract infections.

STANDARD SEQUENTIAL THERAPY

The standard sequential therapy regimen consists of a 5-d dual therapy with a PPI (standard dose, *bid*) and amoxicillin (1 g, *bid*) followed by a 5-d triple therapy with a PPI (standard dose, *bid*), clarithromycin (500 mg, *bid*), and metronidazole/tinidazole (500 mg, *bid*). Recently, several studies have shown that a 10-d sequential therapy

can achieve a promising success rate of 85%-90%[19,33,34]. In a recent rigorous systematic review of 46 randomized clinical trials evaluating 5666 previously untreated patients, Gatta *et al*[35] showed that the overall eradication rate of sequential therapy was 84.3% (95%CI: 82.1%-86.4%) and that sequential therapy was able to eradicate 72.8% (95%CI: 61.6%-82.8%) of the strains resistant to clarithromycin.

This superiority is attributed to the capability of the sequential regimen to overcome clarithromycin resistance. In this regard, a randomized, double-blind, placebo-controlled trial demonstrated that the PP eradication rates of sequential therapy and standard triple therapy for clarithromycin-resistant strains were 89% and 29%, respectively[19].

The mechanism by which sequential administration of antimicrobials is effective despite clarithromycin resistance remains to be fully elucidated. It has been hypothesized that the initial administration of amoxicillin may cause disruption of the bacterial cell wall, thereby preventing the development of clarithromycin efflux channels, which are known to rapidly transport the drug out of the bacterial cell[36]. Alternatively, the improved efficacy of the sequential regimen may be attributed to the larger number of antibiotics (*e.g.*, 3) to which the microorganism is exposed compared with standard triple therapy.

However, more recent studies have questioned both the optimal performance rates and the superiority of sequential therapy compared to legacy triple therapy[37,38]. Indeed, a recent multicenter South American randomized trial demonstrated that 14-d standard triple therapy was more efficacious than the 10-d sequential regimen[17]. Additionally, Choi *et al*[39] showed that, in South Korea, the PP eradication rates of sequential therapy and standard triple therapy were 86% and 77%, respectively, with no statistically significant difference between the two. More recently, a multicenter randomized trial was published comparing high-dose PPI 14-d triple, 14-d sequential, and 10-d sequential therapies in Taiwan[40]. This study showed that 10-d sequential therapy was a nonoptimized regimen, achieving an efficacy of less than 90% in a setting with 9% clarithromycin resistance. As such, the failure of sequential therapy might be expected in settings with high rates of clarithromycin and metronidazole resistance.

MODIFIED SEQUENTIAL THERAPY

Over the past several years, numerous studies have evaluated the use of a modified (*i.e.*, non-clarithromycin-containing) sequential therapy[41].

In 6 studies, the efficacy of a tetracycline-containing sequential therapy was investigated[41]. The eradication rate at ITT analysis varied widely from 50.0% to 87.9%. The eradication rate did not appear to increase when quadruple, rather than triple, therapy was administered for 5 d in the second sequential therapy phase, the cure rate being 78.5%[42]. In 3 studies, a 14-d tetracycline-containing sequential therapy was compared with the 14-d triple therapy. The *H. pylori* eradication rate was higher with the sequential than with the triple regimen, the infection being cured in 77.2% and 63.6% of cases, respectively[43-45]. In 9 studies, the efficacy of a levofloxacin-containing sequential therapy was investigated[41]. The eradication rates at ITT analysis ranged from 65.4% to 96.8%. One study, conducted in an area with > 20% clarithromycin resistance and relatively low (*i.e.*, < 6%) levofloxacin resistance, demonstrated that levofloxacin-containing sequential regimens (at 250 mg *bid* or 500 mg *bid*) were significantly superior to a clarithromycin-containing sequential regimen, achieving eradication rates higher than 90%[46]. This result was confirmed by another study that used high-dose PPI (*i.e.*, esomeprazole 40 mg, *bid*) and high-dose levofloxacin (*i.e.*, 500 mg, *bid*) and demonstrated an eradication rate of 93% at ITT and 95 PP[47]. Levofloxacin-containing sequential regimens have also been demonstrated to achieve cure rates higher than 90% by Molina-Infante *et al*[48], who used levofloxacin 250 mg *bid*, and by Ozdil *et al*[49], who used levofloxacin 500 mg *bid*.

Extending the duration of levofloxacin-containing sequential treatment from 10-d to 14-d does not appear to increase the efficacy, although the 14-d therapy achieved a distinctly higher eradication rate compared with standard 14-d triple therapy (86.6% *vs* 45.3%)[50]. The efficacy of a tinidazole-free sequential therapy was tested in 1 study, in which clarithromycin (500 mg, *bid*) was added to a PPI and amoxicillin (*i.e.*, amoxicillin was not substituted with tinidazole) in the second 5-d segment of the treatment[51]. Following this sequential therapy, the eradication rate did not differ compared to that of a 10-d triple therapy at ITT analysis (73.0% *vs* 72.2%, respectively).

Another study tested the efficacy of two 10-d modified bismuth-containing sequential therapies consisting of a PPI, amoxicillin, bismuth, and metronidazole or clarithromycin for the first 5-d followed by a PPI, amoxicillin, bismuth, and furazolidone for 5 additional days[52]. The infection was cured in 78.5% of cases treated with the metronidazole-containing regimen and in 82% of cases treated with the clarithromycin-containing regimen, the success rates being similar to that of a standard 10-d triple therapy (81.1%).

In conclusion, the value of sequential regimens as first-line therapies for *H. pylori* infection appears to be decreasing, and these regimens will require a thorough re-evaluation.

CONCOMITANT THERAPY

Concomitant therapy is another novel regimen that proved to be successful in the presence of clarithromycin resistance[25]. This 4-drug regimen includes a PPI (standard dose, *bid*), clarithromycin (500 mg, *bid*), amoxicillin (1 g, *bid*), and metronidazole (500 mg, *bid*), all of which are

given for the entire duration of therapy. This therapy is superior to standard triple therapy for *H. pylori* eradication[52] and is also less complex than sequential therapy, as this regimen does not involve changing drugs halfway through. A head-to-head non-inferiority trial of 10-d sequential and 10-d concomitant therapy showed that these therapies were equivalent (93.1% *vs* 93.0% by PP analysis)[53]. Further advantages of concomitant therapy include its simplicity (addition of a nitroimidazole to standard treatment) and its wider geographical validation (including Japan, Colombia, Taiwan, Spain, and Greece) compared with sequential therapy.

Recently, Kim *et al*[54] compared concomitant quadruple therapy with standard triple therapy for first-line *H. pylori* eradication, showing that 5-d quadruple concomitant therapy eradicated *H. pylori* in over 90% of patients. In particular, the eradication rates were 86.1% with the triple therapy and 91.4% with the concomitant therapy (PP); however, the difference was not statistically significant. Georgopoulos *et al*[55,56] recently evaluated the efficacy and tolerability of a 10-d concomitant regimen in Greece. This country has high resistance rates to both clarithromycin (nearly 25%) and metronidazole (approximately 40%)[57,58]; thus, it is a setting in which sequential therapy is reportedly more likely to fail[51]. This was an open-label, single arm trial[55], and thereafter, a randomized controlled trial was performed to compare legacy triple therapy of the same duration (10 d)[57]. The performance of the concomitant regimen at ITT analysis was 90% in the former study and 90.2% (*vs* 73.8% for standard triple therapy) in the latter study.

In a recent comparative study, patients with dual antibiotic resistance had significantly lower eradication rates (*i.e.*, 33.3%) compared with patients without dual resistance (*i.e.*, 95.1%) after sequential therapy, whereas concomitant therapy led to eradication of the infection in 75% of patients with dual resistance compared with 92.4% of patients without dual resistance[53]. However, this study was conducted in a low-clarithromycin-resistance setting where even standard regimens can still yield excellent eradication. In a report from Spain, a country with high rates of antibiotic resistance, a 10-d concomitant therapy successfully eradicated 100% of clarithromycin-resistant and 75% of dual-resistant strains (*vs* 75% and 60%, respectively, with sequential therapy), although the small number of clarithromycin- and dual-resistant strains (5 and 4, respectively), does not allow firm conclusions to be drawn[59]. Recently, our group performed a non-inferiority randomized trial[47] to determine whether a 5-d levofloxacin-containing quadruple concomitant regimen was as safe and effective as the 10-d levofloxacin-containing sequential regimen for eradicating *H. pylori* infection in patients naïve to treatment. ITT analysis showed similar eradication rates for concomitant (92.2%) and sequential therapies (93.3%). The PP eradication results were 96.5% for concomitant therapy and 95.5% for sequential therapy. The differences between the sequential and concomitant treatments were 1.1% in ITT analysis and -1.0% in the PP analysis, confirming that 5-d levofloxacin-containing quadruple concomitant therapy is similarly effective and safe for eradicating *H. pylori* infection compared with 10-d levofloxacin-containing sequential therapy. Additionally, 5-d levofloxacin-containing quadruple concomitant therapy is less expensive than 10-d levofloxacin-containing sequential therapy.

Bismuth-containing quadruple therapy (omeprazole, bismuth, metronidazole, and tetracycline) has been recommended by the Second Asia-Pacific Consensus Guidelines for *H. pylori* Infection[60] and by the Maastricht Ⅳ Consensus Report[28] as an alternative first choice regimen to standard triple therapy in areas with a low clarithromycin resistance, and it is recommended as the first-line therapeutic option in areas with a high prevalence of clarithromycin resistance. Bismuth-containing quadruple therapy has the advantage of utilizing compounds for which resistance has rarely been reported, with the exception of metronidazole; however, metronidazole resistance can be at least partially overcome by increasing the dose and duration of therapy[61]. Two studies, each with more than 100 patients, have demonstrated eradication rates of > 90% when this combination was given for 10 d[44,62]. Recently, a novel bismuth-containing quadruple therapy using a single 3-in-1 capsule containing bismuth subcitrate, metronidazole, and tetracycline has been proposed to decrease the pill burden and improve patient compliance. In a randomized clinical trial, this single-capsule bismuth-containing 10-d treatment showed an ITT cure rate of 80% and a PP cure rate of 94%[18].

In a 2010 meta-analysis evaluating first-line use of 10-d bismuth-containing quadruple therapy or standard therapy, 78.3% of the patients who received quadruple therapy and 77% of those who received standard therapy achieved ITT eradication, indicating similar (and suboptimal) therapeutic effectiveness for both regimens[63]. Recently, Malfertheiner *et al*[18] compared the efficacy of a 10-d bismuth-containing quadruple therapy and a 7-d triple therapy. In this study, quadruple therapy resulted in a PP eradication rate of 94%, whereas triple therapy achieved a rate of only 70%.

Currently, the optimal treatment duration of bismuth-containing quadruple therapy remains unclear; however, a 10-14 d course is most commonly employed in clinics[64].

HYBRID THERAPY

Hsu *et al*[65] reported a hybrid (sequential-concomitant) therapy consisting of a dual therapy with a PPI (standard dose, *bid*) and amoxicillin (1 g, *bid*) for 7 d followed by a concomitant quadruple therapy with a PPI (standard dose, *bid*), amoxicillin (1 g, *bid*), clarithromycin (500 mg, *bid*), and metronidazole (500 mg, *bid*) for 7 d. The new therapy extends the duration of amoxicillin treatment to 14 d and concomitantly employs three antibiotics

Table 1 Recommended first-line therapies for *Helicobacter pylori* infections

	Treatment	Days	No. of patients	Methods of evaluating eradication	Eradication rate % (ITT)	Eradication rate % (PP)	Adverse effects %	Ref.	Type of study
Low clarithromycin resistance area (< 20%)	PPI (standard dose, *bid*) + amoxicillin (1 g, *bid*) + clarithromycin (500 mg, *bid*)	7-10	1975	UBT or H or R	Overall 77.3-100		0-33	Mégraud *et al*[26]	Meta-analysis
	PPI (standard dose, *bid*) + amoxicillin (1 g, *bid*) followed by a triple therapy with a PPI (standard dose, *bid*) + clarithromycin (500 mg, *bid*) + metronidazole/tinidazole (500 mg, *bid*)	5 + 5	5666	UBT or H or R	Overall 84.3		0-44	Gatta *et al*[35]	Meta-analysis
High clarithromycin resistance area (≥ 20%)	PPI (standard dose, *bid*) + amoxicillin (1 g, *bid*) + levofloxacin (250 mg, *bid*)	7-10	900	UBT or H or R	Overall 72-96		0-52	Berning *et al*[32]	Meta-analysis
	PPI (standard dose, *bid*) + amoxicillin (1 g, *bid*) followed by a triple therapy with a PPI (standard dose, *bid*) + levofloxacin (250/500 mg, *bid*) + tinidazole (500 mg, *bid*).	5 + 5	250	UBT	96/96.8	98.3/98.4	22.1-23.5	Romano *et al*[46]	RCT
	PPI (standard dose, *bid*) + amoxicillin (1 g, *bid*) + clarithromycin (500 mg, *bid*) + metronidazole (500 mg, *bid*)	5	135	UBT	91.4	91.4	35.6	Kim *et al*[54]	RCT
	PPI (high dose, *bid*) + amoxicillin (1 g, *bid*) + levofloxacin (500 mg, *bid*) + tinidazole (500 mg, *bid*)	5	90	UBT	92.2	96.5	27.8	Federico *et al*[47]	RCT
	PPI (standard dose, *bid*) + metronidazole (500 mg, *bid*) + bismuth (120 mg, q.i.d.) + tetracycline (500 mg, q.i.d.)	10	218	UBT	92	94	47	Malfertheiner *et al*[18]	RCT
	PPI (high dose, *bid*) + amoxicillin (1 g, *bid*) followed by a quadruple therapy with a PPI (high dose, *bid*) + amoxicillin (1 g, *bid*) + clarithromycin (500 mg, *bid*) + metronidazole (500 mg, *bid*)	7 + 7	171	UBT	90	92	47	Molina-Infante *et al*[66]	RCT

PPI: Proton pump inhibitor; UBT: Urea breath test; H: Histology; R: Rapid urease test; RCT: Randomized controlled trial; PP: Per-protocol; ITT: Intention-to-treat.

in the last 7 d of the treatment course. In 117 *H. pylori* -infected subjects, the novel therapy provided excellent eradication rates of 99% and 97% according to PP and ITT analysis, respectively[65]. It is important to note that the new therapy has a high efficacy for the treatment of *H. pylori* strains harboring dual resistance to clarithromycin and metronidazole. The extension of the amoxicillin treatment duration to 14 d in the hybrid therapy might account for the higher eradication rate of *H. pylori* strains with dual resistance to clarithromycin and metronidazole. Recently, in a randomized clinical trial, Molina-Infante *et al*[66] compared hybrid therapy (omeprazole 40 mg *bid* and amoxicillin 1 g *bid* for 14 d with clarithromycin 500 mg *bid* and nitroimidazole 500 mg *bid* for the final 7 d) with concomitant therapy (the same 4 drugs taken concurrently twice daily for 14 d) in 343 consecutive individuals with *H. pylori* infection who were naïve to treatment and resided in areas of high clarithromycin and metronidazole resistance (Spain and Italy). In PP analysis, the rates of eradication for hybrid and concomitant therapies were 92% and 96.1%, respectively. In ITT analysis, the rates were 90% and 91.7%, respectively, showing that optimized non-bismuth quadruple hybrid and concomitant therapies cured more than 90% of patients with *H. pylori* infections in areas of high clarithromycin and metronidazole resistance. However, further studies comparing bismuth and non-bismuth quadruple regimens in this setting are warranted. Table 1 shows the recommended first-line therapies for *Helicobacter pylori* infections.

CONCLUSION

Ideally, the treatment for an infectious disease should be chosen based on culture and susceptibility testing using biological material (*e.g.*, urine, sputum) obtained from each patient. This is not always feasible in *H. pylori*-infected patients because it requires an invasive procedure

(*i.e.*, esophago-gastro-duodenoscopy), which is not indicated in dyspeptic patients younger than 45 years of age without "alarm" symptoms. To ensure a higher chance of eradicating the infection during the first attempt, an empirical first-line therapy should be chosen based on the pattern of local antimicrobial resistance[67]. We suggest in vitro antimicrobial sensitivity testing for cases in which two different eradication regimens fail to eradicate the infection.

It is important to keep in mind that clarithromycin-containing triple therapy loses efficacy when resistance is between 7% and 10%. Moreover, clarithromycin-containing sequential and concomitant regimens lose efficacy in the face of clarithromycin resistance between 15% and 20% and when metronidazole resistance approaches 40%, thus increasing the likelihood of dual (*i.e.*, clarithromycin + metronidazole) resistance. A valid alternative to concomitant therapy is represented by hybrid (*i.e.*, sequential + concomitant) therapy, which has proven to be effective in more than 90% of *H. pylori*-infected patients in the setting of high clarithromycin and metronidazole resistance[66]. Bismuth-containing quadruple therapy is also a valid alternative; however, in our opinion, the duration should be extended to 14 d to overcome metronidazole resistance.

Compliance is an important issue, and significant effort should be directed toward identifying a regimen that is short and easy for the patient to follow. In this regard, the recently reported 5-d levofloxacin concomitant regimen[47] might represent an easy regimen to follow, although its use as an empirical first-line therapy should be limited to patients living in areas where fluoroquinolone resistance is rare and resistance to both clarithromycin and metronidazole is high. Additionally, the 5-d levofloxacin concomitant regimen might be regarded to as a good alternative to rescue therapy, provided the patient has no history of prior fluoroquinolone resistance[68].

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (6): *Helicobacter pylori*

Probiotics for the treatment of *Helicobacter pylori* infection in children

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Abstract

The combination of a proton pump inhibitor and two antibiotics (clarithromycin plus amoxicillin or metronidazole) has been the recommended first-line therapy since the first guidelines for *Helicobacter pylori* (*H. pylori*) infection in children were published. In recent years, the success of eradication therapies has declined, in part due to the development of *H. pylori* resistant strains. Alternative anti-*H. pylori* treatments are currently becoming more popular than the traditional eradication methods. Components that may be used either as a monotherapy or, in combination with antimicrobials, resulting in a more effective anti-*H. pylori* therapy have been investigated in depth by several researchers. One of the potential therapies is probiotic cultures; promising results have been observed in initial studies with numerous probiotic strains. Nevertheless, many questions remain unanswered. In this article, we comprehensively review the possible mechanisms of action of probiotics on *H. pylori* infection, and present the results of published studies using probiotics as possible agents to control *H. pylori* infection in children. The effect of the addition of probiotics to the standard *H. pylori* eradication therapy for the prevention of antibiotic associated side-effects is also discussed.

Key words: *Helicobacter pylori*; Children; Probiotics; Eradication treatment; Prevention

Core tip: Because of the decrease in the *Helicobacter pylori* (*H. pylori*) eradication rate after standard triple therapy with a proton pump inhibitor and two antibiotics, alternative therapies have recently received attention. In this article, we comprehensively review the possible mechanisms of action of probiotics on *H. pylori* infection, and present the results of the published studies using probiotics as possible agents to control *H. pylori* growth in children. The effect of the addition of probiotics to the standard *H. pylori* eradication therapy for the prevention of antibiotic associated side-effects is also discussed.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a highly prevalent, serious and chronic infection that has been associated causally with a diverse spectrum of gastrointestinal disorders including chronic gastritis, peptic ulcer disease, gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue lymphoma[1]. In both developed and developing countries, *H. pylori* is most frequently acquired during childhood, and is associated with family size, clustering in families, low socioeconomic status and low level of education[2-5]. It is commonly thought that once the *H. pylori* infection is acquired, it evolves toward persistent chronic infection[6] and that spontaneous clearance is relatively rare[6-8]. However, in a study of children in which prevalence by age was reported in intervals of 1 year, no increase in prevalence by age was observed[9]. This suggests that transient *H. pylori* infection is not uncommon in children[6,10,11]. In 6-24-mo old children in Mexico, and Texas, researchers found 80% spontaneous reversion of the infection[10,11]. In a very recent study involving 718 schoolchildren in Mexico City, Duque *et al*[12] found that the majority of them maintained their initial status of *H. pylori* infection throughout the follow-up, while 11.7% showed changes in their infection status. Variables related to health status and infection transmission, such as iron status and number of siblings, were shown to be important for the incidence of *H. pylori* and the spontaneous clearance of infection[12].

The combination of a proton pump inhibitor (PPI) and two antibiotics (clarithromycin plus amoxicillin or metronidazole) has been the recommended first-line therapy since the first guidelines for *H. pylori* infection in children were published[13-15]. In recent years, the success of eradication therapies has declined, in part due to the development of *H. pylori* resistant strains[16]. Several studies have documented high resistance rates to clarithromycin and metronidazole in paediatric and adult populations[17-19]. In Europe, Koletzko *et al*[17] showed that primary resistance to clarithromycin and metronidazole was present in 20% and 23% of *H. pylori* strains respectively, while secondary resistance was found in 42% and 35% of the strains recovered after at least one failed treatment for *H. pylori*. The use of clarithromycin for other indications, mainly for respiratory tract infections, seemed to be the major risk factor for development of primary resistance to this drug. On the other hand, the only risk factor for primary metronidazole resistance was immigration from a non-European country. In fact, the authors showed that children born in Asia, Africa or the Middle East had a 2.4 times higher risk for primary metronidazole resistance than patients of the same age and gender born in Europe. Iterative metronidazole treatments for parasitic or diarrhoeal diseases in children originating from Africa and Asia may be incriminated in the increased primary resistance rates of metronidazole recorded in these paediatric populations. A prospective United States multicentre study in adults and children also documented similar high clarithromycin resistance rates[18]. Declining eradication rates with standard triple regimens have led to the development of alternate treatment options[20,21].

Recently, ESPGHAN and NASPGHAN jointly renewed clinical guidelines for *H. pylori* infection in children using a standardised evidence based approach[22]. Bismuth-based triple therapy or sequential therapy was recommended as alternate first-line regimens. Quadruple therapy with PPI, metronidazole, amoxicillin, and bismuth was also suggested as second line therapy or salvage therapy in the absence of primary culture and sensitivity testing[20,22]. These regimens have the disadvantages of being expensive, risking poor compliance, causing side-effects and encouraging the emergence of resistance. Moreover, as most of the colonized children remain asymptomatic, the administration of antibiotic treatments is not ethically acceptable. Other factors limiting the administration of such treatments in developing countries is their high cost for families from low socio-economic strata (those most affected by the infection) and the relative inefficiency of the antibiotics due to the fact that children tend to be rapidly re-colonized. Alternative anti-*H. pylori* treatments are currently becoming more popular than the traditional eradication methods. Components that may be used either as a monotherapy or, in combination with antimicrobials, resulting in a more effective anti-*H. pylori* therapy have been investigated by several researchers[23]. One of the potential therapies involves probiotic cultures; promising results have been observed in initial studies with numerous probiotic strains[24-26]. Nevertheless, many questions remain unanswered. In this article, we comprehensively review the possible mechanisms of action of probiotics on *H. pylori* infection, followed by the outcomes of the published studies using probiotics as possible agents to control *H. pylori* growth in children. The effect of the addition of probiotics to the standard *H. pylori* eradication therapy for the prevention of antibiotic associated side-effects is also discussed.

DEFINITION

Probiotic

An oral supplement or a food product that contains a sufficient number of viable micro-organisms to alter the microflora of the host and has the potential for beneficial health effects[27,28].

Probiotic micro-organisms are typically members of the genera *Lactobacillus*, *Bifidobacterium*, and *Streptococcus*[27-29]. These bacteria are fermentive, obligatory, or facultative anaerobic organisms, which are typically non-motile and of varying shapes. Typically they produce lactic acid. Their inherent biological features enable them to predominate and prevail over potential pathogenic microorganisms in the human digestive tract. It is currently hypothesized that these microbes generate small molecular metabolic byproducts that exert beneficial regulatory influence on host biological functions, including short-chain fatty acids such as butyrate. These metabolic

byproducts are sometimes referred to as "postbiotics" and may function biologically as modulators of immune function[30]. The most studied probiotic bacteria to date belong to the genera *Lactobacillus* and *Bifidobacterium*. Some yeasts and yeast byproducts have also been studied and have been used as probiotic agents, for example the yeast *Saccharomyces boulardii*.

MECHANISMS OF ACTION

Several probiotic strains, especially *lactobacilli,* have exhibited antagonistic properties against *H. pylori in vitro*[31]. There are several putative mechanisms for probiotic efficacy against *H. pylori*. *Lactobacilli* species are commensal in the human alimentary tract and their concentrations in the normal stomach vary between 0 and 10^3/mL fluid[25]. Being acid resistant, they persist in the stomach longer than other bacteria. The possible role of the local microbiota in the protection against gastric lesions is suggested by the study of Elliott *et al*[32] who found that the level of total aerobes in the stomach of healthy rats ranged from 10^3 to 10^4 CFU/g of tissue, with Gram-negative micro-organisms representing only 5% of the population; autochthonous gastric *lactobacilli* were present in all rats. However, one day after the induction of gastric ulcers the total aerobe count peaked at 10^9-10^{10} CFU/g and remained high for 1 wk. At this time, Gram-negative bacteria were the majority of the total aerobes while the *lactobacilli* population disappeared. Colonization by Gram-negative bacteria occurred preferentially at the site of ulcer. These findings suggest that the gastroduodenal microbiota, though low numerically, could represent a first line of defense against pathogenic bacteria. Thus, the intake of exogenous lactic acid bacteria, in particular those with probiotic properties, may reinforce these protective functions in the stomach by maintaining local microbiological homeostasis, interfering with *H. pylori* and/or decreasing inflammatory processes[31].

Non-immunological barriers such as the acidity of the stomach and the gastric mucosal barrier also represent a first line of defense against pathogenic bacteria. Two main types of substances have been implicated in the inhibition of *H. pylori* by lactic acid bacteria: short chain fatty acids (SCFAs) and bacteriocins. SCFAs such as acetic, propionic, butyric, and lactic acids are produced during the metabolism of carbohydrates by probiotics and have an important role in decreasing pH[33]. Bhatia *et al*[34] were the first to observe an antagonistic effect of a lactobacillus strain against *H. pylori* and to implicate SCFAs in this effect. A dose-dependent inhibition of *H. pylori* growth has been observed with acetic and lactic acid, the latter demonstrating the most intense effect[35]. Lactic acid, in addition to its antimicrobial effect resulting from the lowering of the pH, could inhibit the *H. pylori* urease. However, the inhibitory effects of *lactobacilli* on *H. pylori* differ from strain to strain[23]. Certain *lactobacilli* synthesize antimicrobial compounds related to the bacteriocin family[36,37]. Bacteriocins are compounds with potential anti-*H. pylori* activity. They are small, heat-resistant and dialysable peptidic structures with antimicrobial activities, which are synthesized by several bacterial species including lactic acid bacteria[23].

Other possible mechanisms of protection induced by probiotics include inhibition of the adhesion of *H. pylori*. The adhesion of *H. pylori* to epithelial cells is important in determining the outcome in *H. pylori*-associated diseases[38]. Certain *lactobacilli* can exert their antiadhesion activity by secreting antimicrobial substances[23]. However, strains such as *lactobacilli reuteri* (*L. reuteri*) can inhibit *H. pylori* growth by competing with adhesion receptors[39]. A nonspecific rather than a specific blockage of receptor sites is the most likely mechanism because *lactobacilli* can inhibit adhesion of a large varieties of pathogenic bacteria, although each adheres to its particular receptor on the cells[40].

It has been suggested that intake of probiotics strengthens the mucosal barrier by stimulating mucin production. Reduced mucus secretion in a damaged epithelium is a frequent finding in *H. pylori*-associated gastritis. *H. pylori* is known to suppress MUCI and *MUC5A* gene expression in a human gastric cell line[41]. It has been shown *in vitro* that *lactobacilli plantarum* and *lactobacilli rhamnosus* increase the expression of *MUC2* and *MUC3* genes[42]. This property can mediate the ability of these strains to restore the mucosal permeability of gastric mucosa[43] or inhibit the adherence of pathogenic bacteria, including *H. pylori*[42].

Finally, modulation of immune response to pathogens should also be taken into account as a potential mechanism of probiotic efficacy. The inflammatory response to gastric *H. pylori* infection is characterized by the release of various inflammatory mediators such as chemokines and cytokines[23]. Probiotics could modify the immunologic response of the host by interacting with epithelial cells and modulating the secretion of antiinflammatory cytokines, which would result in a reduction of gastric activity and inflammation[44]. However, the effect of probiotics on the immune response is difficult to generalize. Distinct probiotics strains may generate different immune responses, which, in turn, depend on the host's immune status[45].

PROBIOTICS AND *H. PYLORI* INFECTION

Several studies using murine models have shown that probiotic treatment, although it is unable to clear *H. pylori*, is effective in reducing bacterial colonization and decreasing gastric inflammation in *H. pylori*-infected mice[46-50]. It has been postulated, on the basis of the results of *in vitro* and animal studies, that probiotics could possibly compete with and down-regulate *H. pylori* infection in humans[23]. Though utilization of probiotics alone does not lead to the eradication of *H. pylori*[51-55], a growing body of recent evidence suggests that regular intake of probiotics suppresses *H. pylori* infection in humans, maintaining lower levels of this pathogen in the stomach[25].

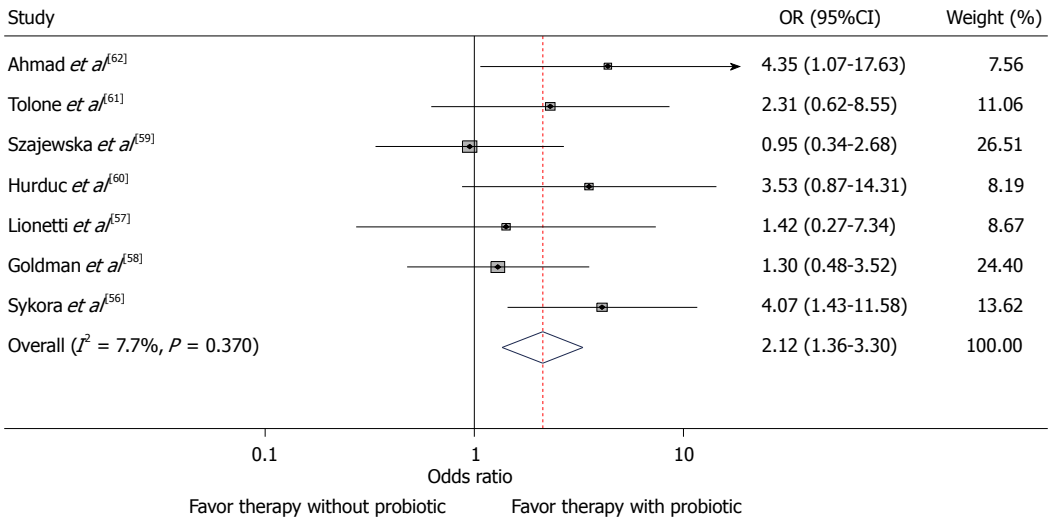

Figure 1 Forest plot showing odds ratios obtained from seven trials comparing an antibiotic therapy with the same therapy plus a probiotic. The antibiotic therapies and the probiotics are not the same in all trials.

Few clinical trials evaluating the use of different probiotic strains have been reported. In some of these studies, probiotics were used alone while in others they were used as adjunctive agents in the classical treatment of *H. pylori* infection.

Utilization of probiotics in association with antibiotics in the treatment of H. pylori

The clinical trials performed in children on the effect of probiotics on *H. pylori* eradication rates as an adjuvant to eradicating regimens are summarized in Table 1 and Figure 1. In the earliest study, Sýkora *et al*[56] found that the addition of *lactobacilli casei* (*L. casei*) DN-114001 to a standard triple therapy improved the rate of *H. pylori* eradication. Intention-to-treat based eradication rates for the triple therapy group supplemented with *L. casei* were 84.6% (95%CI: 71.2%-95.5%), and 91.6% (95%CI: 76.9%-98.2%) by per-protocol analysis. Eradication in the placebo group was 57.5% (95%CI: 42.2%-72.3%) in the intention-to-treat analysis and 61.3% (95%CI: 44.4%-75.0%) in the per protocol analysis. Reported adverse effects were infrequent and self-limiting after therapy cessation in both groups[56].

In a randomized, double-blind, controlled trial conducted in Italy[57], symptomatic children with *H. pylori* infection were treated with 10-d sequential therapy and randomized to receive either *L. reuteri* ATCC 55730 or placebo for 20 d. All children (or family member) also attended an interview to recall history of gastrointestinal symptoms and the 15-item Gastrointestinal Symptom Rating Scale (GSRS) was used to assess severity and frequency of symptoms. The following symptoms were specifically investigated: epigastric burning and/or pain, abdominal pain, acid regurgitation, heartburn, sucking sensation in the epigastrium, nausea, vomiting, bloating, abdominal distension, eructation, increased flatus, disorders of defecation, inappetence, halitosis, taste disturbance and urticaria. The symptoms were scored by the child (or family member) on a four-point scale: mild (non-interfering with daily activities), moderate (slightly interfering with daily activities), severe (interfering with daily activities), very severe (continuous and if on therapy, producing treatment interruption). Stool consistency was graded from hard (0) to watery (4). Data were collected before (1 wk before intervention), during (5th and 10th day) and after completion of eradicating therapy (15th and 20th day) and patients were invited to return their diaries immediately after the intervention period. No significant difference in *H. pylori* eradication rates between the treated group and the control group were found. However, in all probiotic supplemented children when compared with those receiving placebo there was a significant reduction in GSRS score during eradication therapy which became markedly evident at the end of follow-up (Table 1). Children receiving *L. reuteri* reported less side-effects than those receiving placebo[57].

Goldman *et al*[58] tested the efficacy of a commercial yogurt containing *B. animalis* and *L. casei* as an adjuvant to triple therapy and found no significant difference in *H. pylori* eradication rates at 1 and 3 mo between probiotic and placebo group. Side effects were not assessed. Similarly, in a randomized, double-blind, controlled trial conducted in Poland[59], no difference was found with respect to *H. pylori* eradication rates between children who received triple therapy supplemented with *Lactobacillus GC* and the control group. Also, the incidence of adverse effects was not reduced. In a randomized, open trial conducted in Romania[60], children with dyspepsia and *H. pylori* infection were treated with eradication triple therapy and randomized to receive either *Streptococcus boulardii* (*S. boulardii*) (for 4 wk) or placebo. No significant difference in *H. pylori* eradication rates between the treated group and the control group were found. However, the incidence of side effects was reduced in the *S. boulardii* group.

Recently, Tolone *et al*[61] supplemented a standard triple

Table 1 Clinical trials in children using probiotics as a complement during *Helicobacter pylori* eradication treatment

Ref.	Study design	Therapy	Probiotic strain (product; dose; time)	Patients	Diagnosis	No. of treated patients	Eradication *n* (%); *P* value	Side effects *n* (%); *P* value	Test for confirming eradication (time after completion of therapy)
Sýkora *et al*[56]	P, R, DB, PC	A (25 mg/kg twice daily), C (7.5 mg/kg twice daily), and O (10 or 20 mg twice daily) 1 wk + probiotic *vs* same eradication therapy + placebo	*L. casei* DN-114 001 10^{10} CFU in 100 mL of fermented milk (actimel, Danone); 2 wk	86 (aged 9-15 yr) symptomatic children and adolescents	EGDS (histopathology, culture, and RUT) and HpSA	39[1] 36[2] *vs* 47[1] 44[2]	33 (84.6)[1] 33 (91.6)[2] *vs* 27 (57.5)[1]; 0.0045 27 (61.3)[2]; 0.0019	9 (23.1) *vs* 10 (21.2); NS (nausea, headache, abdominal pain, recurrent vomiting, diarrhoea)	HpSA and ^{13}C-UBT (4 wk)
Gotteland *et al*[64]	O, R	A (50 mg/kg tid), C (15 mg/kg bid), and L (1 mg/kg bid) 8 d *vs* Probiotic *vs* Symbiotic	*Lactobacillus acidophilus LB* (LB); capsule containing 10^9 heat-killed and lyophilized LB (Lacteol Forte, Laboratoire du Dr. Boucard, Paris, France); b.i.d. for 8 wk, and *Saccharomyces boulardii* plus inulin (Sbl); sachet containing 250 mg of lyophilized Sb (Perenteryl, Merck Quìmica Chilena, Santiago, Chile); bid for 8 wk	141 (aged 5-12 yr) asymptomatic children. 81 children were observed without any treatment	^{13}C-UBT	45[2] *vs* 46[2] *vs* 50[2]	30 (66)[2] *vs* 3 (6.5)[2] *vs* 6 (12)[2]; < 0.001 No spontaneous clearance was observed in children without treatment	NA	^{13}C-UBT (1 d)
Goldman *et al*[58]	R, DB, PC	A (50 mg/kg per day), C (20 mg/kg per day bid), and O (1 mg/kg per day) 1-wk + probiotic *vs* same eradication therapy + placebo	*Bifidobacterium animalis* and *Lactobacillus casei* (10^7 CFU/mL) in 250 mL of a commercial yogurt; once daily for 3 mo	65 (aged 5-15 yr) symptomatic children and adolescents	EGDS and ^{13}C-UBT (histological data NA)	33[1,2] *vs* 32[1,2]	15 (45.5)[1,2] *vs* 12 (37.5)[1,2]; 0.345 at 1 mo 14 (42.4)[1,2] *vs* 13 (40.6)[1,2]; 0.542 at 3 mo	NA	^{13}C-UBT (1 and 3 mo)
Lionetti *et al*[57]	R, DB, PC	O (1 mg/kg/die) plus A (50 mg/kg/die) for 5 d followed by O (1 mg/kg/die) plus C (15 mg/kg/die)and T (20 mg/kg/die) for the next 5 d + probiotic *vs* same eradication therapy + placebo	*L. reuteri* [pill containing 10^8 CFU of *L. reuteri* ATCC 55730 (SD2112), Reuterin, Nòos]; one pill once daily for a period of 20 d	40 (aged 3.3-18 yr) symptomatic children and adolescents	EGDS (histopathology and RUT) [pangastritis (27); antral gastritis, mild (20); antral gastritis, moderate (14); antral gastritis, severe (10)]	20[1,2] *vs* 20[1,2]	17 (85)[1,2] *vs* 16 (80)[1,2]; NS	Reduction of GSRS score during eradication therapy [4.1 ± 2 (95%CI: 2.9-5.9) *vs* 6.2 ± 3 (95%CI: 5.2-8.3); $P < 0.01$] and at the end of follow-up [3.2 ± 2 (95%CI: 2.4-4) *vs* 5.8 ± 3.4 (95%CI: 4.8-6.9); $P < 0.009$]; Epigastric pain (15% *vs* 45%; $P < 0.04$); Abdominal distension (0% *vs* 25%; $P < 0.02$); Eructation (5% *vs* 35%; $P < 0.04$); Disorders of defecation (15% *vs* 45%; $P < 0.04$); Halitosis (5% *vs* 35%; $P < 0.04$)	^{13}C-UBT (8 wk)

Hurduc *et al*[60]	O, R	A (50 mg/kg per day, bid) and C (15 mg/kg per day, bid) 7-10 d; O or E (1 mg/kg per day, bid) 3-wk + probiotic *vs* same eradication therapy + placebo	*Saccharomyces boulardii*, Enterol, Biocodex, Gentilly Cedex; 250 mg bid; 4-wk	90 (aged 3-18 yr) children and adolescents with dyspepsia	EGDS (histopathology and RUT) [chronic gastritis: mild (8); moderate-to-severe (82); active (32); inactive (58)]	48[1,2] *vs* 42[1,2]	45 (93.3)[1,2] *vs* 34 (80.9)[1,2]; NS	4 (8.3) *vs* 13 (30.9); $P = 0.047$ (bloating, taste disturbance, nausea, abdominal pain, diarrhoea, constipation, loss of appetite, fatigue)	EGDS (4-6 wk) (histopathology and RUT)
Szajewska *et al*[59]	R, DB, PC	A (50 mg/kg per day bid), C (20 mg/kg per day bid), and O (1 mg/kg per day) 1-wk + probiotic *vs* same eradication therapy + placebo	*Lactobacillus* GG 1 × 10^9 CFU; 7 d	83 (aged 5-17 yr) symptomatic children and adolescents. Excluded from the analysis were 17 children for lack of diary and/or ^{13}C-UBT	EGDS (2 of 3 tests - ^{13}C-UBT, histopathology or RUT) [histological data NA]	34[2] *vs* 32[2]	23 (69)[2] *vs* 22 (68); RR = 0.98 (95%CI: 0.7-1.4)[2]	Therapy-related diarrhea: 2 (6) *vs* 6 (20); P = NS Total side effects: 18 (51.4) *vs* 13 (40.6); P = NS Abdominal pain: 0 *vs* 0 Nausea: 4 (11.4) *vs* 3 (9.4); P = NS Vomiting: 2 (5.7) *vs* 1 (3.1); P = NS Constipation: 2 (5.7) *vs* 2 (6.2); P = NS Flatulence: 3 (8.6) *vs* 1 (3.1); P = NS Taste disturbance: 4 (11.4) *vs* 5 (15.6); P = NS Loss of appetite: 3 (8.6) *vs* (3.1); P = NS Need for discontinuation of therapy: 0 *vs* 0	^{13}C-UBT (4 wk)
Tolone *et al*[61]	R	A (50 mg/kg per day bid), C (15 mg/kg per day bid), and O (1mg/kg per day) 1-wk + probiotic *vs* same eradication therapy + placebo	*Lactobacillus Plantarum* 5 × 10^9, *L. reuterii* 2 × 10^9, *L. casei subsp. Rhamnosus* 2 × 10^9, *Bifidobacterium infantis* and *B. longum* 2 × 10^9, *L. salivarius* 1 × 10^9, *L. acidoPhilus* 1 × 10^9, *Streptococcus termophilus* 5 × 10^9, and *L. sporogenes* 1 × 10^9 + inuline as a prebiotic (5 g/dayose q.d., Probinul , Cadigroup); 7 d	68 (mean age, 8.3 yr) children with heartburn, dyspepsia, nausea and epigastric pain	EGDS (histopathology) [histological data: NA]	34[1,2] *vs* 34[1,2]	30 (88.2)[1,2] *vs* 26 (76.4)[1,2]; 0.1	Epigastric pain: 2 (5.8) *vs* 6 (17.6); $P < 0.05$ Nausea: 1 (2.9) *vs* 3 (8.8); $P < 0.05$ Vomiting : 0 *vs* 2 (5.8); $P < 0.05$ Diarrhea: 0 *vs* 8 (23.5); $P < 0.05$	^{13}C-UBT (4 wk)
Ahmad *et al*[62]	R, DB, PC	A (50 mg/kg per day bid) and F (6 mg/kg per day bid) 1-wk; O (1 mg/kg per day) 4-wk + probiotic *vs* same eradication therapy + placebo	*Lactobacillus acidophilus, L. rhamnosus, L. bulgaricus, L. casei, Streptococcus thermophilus, Bifidobacterium infantis, B. breve*; 1 × 10^9 CFU/1 sachet, Protexin Co; 4 wk	66 (aged 3-14 yr) children with chronic abdominal pain, gastrointestinal bleeding, unexplained frequent vomiting and unexplained iron deficiency anemia	EGDS (positive RUT or histopathology) [Antral nodularity (57); Gastric erythema (16); Duodenal ulcer (14); Gastric ulcer (1)]	33[1,2] *vs* 33[1,2]	30 (90.1)[1,2] *vs* 23 (69.7)[1,2]; 0.04	ConstiPation: 2 (5.8) *vs* 2 (5.8); P = NS Nausea/vomiting: 2 (6.1) *vs* 9 (27.3); $P = 0.02$ Diarrea: 2 (6.1) *vs* 8 (24.2); $P = 0.04$ Abdominal bloating: 3 (9.1) *vs* 4 (12.1); $P = 1$	HpSA (4-8 wk)

[1]Intention-to-treat analysis; [2]Per-protocol analysis. O: Open; R: Randomized; DB: Double-blind; SB: Single blind; PC: Placebo controlled; P: Prospective; A: Amoxicillin; C: Clarithromycin; F: Furazolidone ; M: Metronidazole; T: Tinidazole; O: Omeprazole; E: Esomeprazole; L: Lansoprazole; CFU: Colony forming units; RUT: Rapid urease test; UBT: Urea breath test; *H. Pylori*: *Helicobacter pylori*; HpSA: *H. Pylori* stool antigens; GSRS: Gastrointestinal symptom rating scale; NA: Not available; *L. johnsonii*: *Lactobacilli johnsonii*; *L. paracasei*: *Lactobacilli paracasei*.

therapy with a commercial probiotic for 7 d and showed that there was no improvement in the rate of *H. pylori* eradication in the probiotic group. However, the addition of probiotic to triple therapy significantly decreased the frequency of epigastric pain, nausea, vomiting, and diarrhea. In a more recent double-blind randomized placebo controlled study[62], *H. pylori*-positive children were treated with a triple drug treatment protocol and randomly allocated to receive either probiotic or placebo. *H. pylori* was eradicated in 90.09% of patients receiving probiotic and in 69.69% of those receiving placebo ($P = 0.04$). In probiotic supplemented children there was a lower rate of nausea/vomiting and diarrhea.

In summary, seven of the eight studies listed in Table 1 compare eradication rates for groups treated with antibiotics with those treated with antibiotics plus probiotics. The odds ratios for these seven studies are shown in the forest plot in Figure 1. Six of these seven have estimated odds ratios greater than 1.0 implying an estimated benefit for the addition of probiotics, but only two are statistically significant. The antibiotics used in the studies differ as do the treatment regimens. Similarly, the probiotics used and diagnostic techniques differ between studies. With such heterogeinity of design, even though the statistical test of the heterogeneity is not significant ($\chi^2 = 6.5$; $P = 0.37$), a meta-analysis of these studies would not be appropriate.

Utilization of probiotics alone

The clinical trials performed in children on the effect of probiotics on *H. pylori* eradication rates alone are summarized in Table 2. In a double-blind, randomized, controlled clinical trial Cruchet *et al*[63] evaluated the efficacy of *lactobacilli johnsonii* (*L. johnsonii*) La1 or *lactobacilli paracasei* (*L. paracasei*) ST11 as a unique intervention on *H. pylori* eradication in 252 asymptomatic school children screened for *H. pylori* by ^{13}C-Urea breath test (UBT). Subjects were distributed into five groups to receive a product containing live *L. johnsonii* La1 or *L. paracasei* ST11, heat-killed *L. johnsonii* La1 or *L. paracasei* ST11, or just vehicle everyday for 4 wk. There was a moderate but significant difference in ^{13}C-UBT values in children receiving live *L. johnsonii* La1, whereas no differences were observed in the other groups. The authors conclude that regular ingestion of a probiotic strain such as *L. johnsonii* La1 may interfere with *H. pylori* colonization in asymptomatic children and may be an effective alternative to modulate *H. pylori* infection and its associated gastritis in pediatric populations with high prevalences of infection by this pathogen.

In a randomized open trial, Gotteland *et al*[64] randomized asymptomatic *H. pylori*-positive children to receive either 7-d triple therapy, or *Saccharomyces boulardii* as a symbiotic simultaneously with inulin or *L. acidophilus* LB daily for 8 wk. An additional group of asymptomatic *H. pylori*-positive children was followed for 8 wk without any treatment. A significant decrease in ^{13}C-UBT, performed after 8 wk, was observed in the antibiotic group and in the *S. boulardii* group but not in the *L. acidophilus* LB group. No changes in ^{13}C-UBT values were observed in untreated children. The results of this study suggest that the suppressive effect on *H. pylori* colonization in children depends on the probiotic strain used.

In a multicentric, randomized, controlled, double-blind trial carried out in 271 asymptomatic children who tested positive for *H. pylori* by ^{13}C-UBT, Gotteland[65] evaluated whether cranberry juice and the probiotic *L. johnsonii* La1 could act additively or synergistically to suppress *H. pylori*. Subjects were allocated in four groups: cranberry juice/*L. johnsonii* La1, placebo juice/*L. johnsonii* La1, cranberry juice/heat-killed *L. johnsonii* La1, and placebo juice/heat-killed *L. johnsonii* La1 (control), given for 3 wk, after which a second UBT was carried out. A third ^{13}C-UBT was done after one-month washout in those children who tested negative in the second ^{13}C-UBT. *H. pylori* eradication rates significantly differed in the four groups: 1.5% in the control group compared with 14.9%, 16.9%, and 22.9% in the placebo juice/*L. johnsonii* La1, cranberry juice/heat-killed *L. johnsonii* La1, and cranberry juice/*L. johnsonii* La1, respectively; the latter group showed a slight but not significant increase when compared with the other treated groups. The third ^{13}C-UBT was carried out only in 19 of the 38 children who tested negative in the second ^{13}C-UBT and *H. pylori* was detected in 80% of them, suggesting just a temporary inhibition of the organism that disappeared once the administration of the inhibiting factors was interrupted.

In a recent study *L. gasseri* OLL2716 was administered in cheese to pre-school children to evaluate whether its long time administration (for one year) can eradicate *H. pylori* and/or prevent *H. pylori* infection[66]. A total of 440 children were screened by the *H. pylori* stool antigen (HpSA) test. Thereafter, 132 *H. pylori*-positive and 308 *H. pylori*-negative children were recruited to eradication and randomized prevention arms, respectively. Of the 132 *H. pylori*-positive children, 28 withdrew in the beginning because they did not like the cheese. However, 18 of the 28 subjects agreed to undergo an HpSA test again 1-year later, and were designated as the control group. Eighty-two of the remaining *H. pylori*-positive subjects completed the eradication arm, of which 24 (29.3%) were considered to be cured after treatment according to the HpSA test, whereas no eradication was observed in the six subjects in the placebo group consuming ordinary cheese. Spontaneous eradication was found in 1 of 18 children (5.6%) who represented the control group. The difference in the rate of eradication between the active and control groups was statistically significant. However, HpSA test was repeated in 12 of 24 subjects who were HpSA- negative after undergoing the *L. gasseri* treatment, but found that 5 of those 12 (41.7%) had reversed to be HpSA-positive. Therefore, a final eradication rate was around 17%. In the randomized prevention arm, 123 of 156 (79.0%) and 99 of 122 (81.0%) completed active and placebo arms, respectively, of which 4.1% and 8.1% were HpSA positive at 12 mo based on a per-protocol analysis ($P = 0.21$).

Table 2 Clinical trials using probiotics in the treatment of *Helicobacter pylori* infection in children

Ref.	Study design	Probiotic strain (product; dose; time)	Patients	Diagnosis	Eradication *n* (%); *P* value	Test for confirming eradication (time after completion of therapy)	Comments
Cruchet *et al*[63]	DB, R	*Lactobacillus johnsonii* (La1), living or heat-killed, 80 mL/die, (> 10^7 CFU/mL) (Chamyto, Nestlé) and *L. helveticus* (LH) for 4 wk; *Lactobacilli paracasei* ST11, living or heat-killed, (> 10^7 CFU/mL) and LH for 4 wk	252 (aged 6-17 yr) asymptomatic children and adolescents: Living La1/LH, $n = 51^1$; Heat-killed La1/LH, $n = 50^1$; Living ST11/LH, $n = 50^1$; Heat-killed ST11/LH, $n = 51^1$; LH, $n = 50^1$	^{13}C-UBT	A moderate but significant difference in ^{13}C-UBT values was detected in children receiving live La1, whereas no differences were observed in the other groups	^{13}C-UBT (at the end of treatment)	
Gotteland *et al*[65]	R, DB, PC	*Lactobacillus johnsonii* La1, living or heat-killed, 80 mL/die, (> 10^7 CFU/mL) for 3 wk (Chamyto, Nestlé) with or without cranberry juice (CB) (200 mL)	271 (aged 6-16 yr) asymptomatic children and adolescents: CB/La1, $n = 70^1$; Placebo juice/La1, $n = 67^1$; CB/heat-killed La1, $n = 65^1$; Placebo juice/heat-killed La1 (control), $n = 69^1$	^{13}C-UBT	16 (22.9)[1] 11 (16.9)[1] 10 (14.9)[1] *vs* 1 (1.5)[1]; $P < 0.01$	^{13}C-UBT (a second ^{13}C-UBT at the end of treatment and a third ^{13}C-UBT after 1 mo)	The third UBT was carried out in only 19 of the 38 children found to be *H. Pylori*-negative in the second UBT: 12, 2, and 5 subjects from the CB/La1, placebo juice/La1, and CB/heat-killed La1 groups, respectively. Only four children (21) remained negative, after 1 mo without treatment: two from the placebo juice/La1 group and two from the CB/La1 group
Boonyaritichaikij *et al*[66]	SB, PC	*Lactobacillus gasseri* OLL2716 (LG21), pieces of cheese weighing 1.6-2.0 g, approximately 5 × 10^8 CFU/g for 1 yr	88 (aged 3-7 yr) asymptomatic children and adolescents completed the eradication arm: LG21, $n = 82^1$; ordinary cheese, $n = 6^1$ while 222 completed the prevention arm: LG21, $n = 123^1$; Ordinary cheese, $n = 99^1$	HpSA	24 (29.3)[1] *vs* 0. In the randomized prevention arm: 5 (4.1) *vs* 8 (8.1); $P = 0.21$ were HpSA positive at 12 mo	HpSA (1 yr)	A total of 440 asymptomatic children were screened by the HpSA test. Thereafter 132 H. Pylori positive and 308 *H. Pylori* negative children were recruited to eradication and randomized prevention arms, respectively. Eradication was defined as reversion by HpSA at 12 mo; prevention as persistently HpSA negative at 12 mo

[1]Per-protocol analysis. R: Randomized; DB: Double-Blind; SB: Single Blind; PC: Placebo Controlled; CFU: Colony Forming Units; ^{13}C-UBT:Urea Breath Test; *H. Pylori*: *Helicobacter pylori*; HpSA: *H. Pylori* stool antigens.

CONCLUSION

So far, there has been no convincing evidence on the beneficial effect of supplementation of probiotics to triple therapy for eradicating *H. pylori* infection in children. The very few trials performed in children on the effect of probiotics alone suggest just a temporary inhibition of *H. pylori* that disappears once the administration of the inhibiting factors are interrupted. Nonetheless, the majority of these studies were based on relatively small samples and, therefore, they may lack the statistical power necessary to detect an important effect of the probiotics. Finally, in most studies, the effect of probiotic treatment on *H. pylori* infection in children has been estimated indirectly by ^{13}C-UBT. On the other hand, probiotic treatment seems to be able to reduce *H. pylori* therapy associated side effects and indirectly may help to improve the eradication rate; however it seems that the beneficial effects are strain specific. We conclude that standardized multicenter, placebo-controlled studies in larger series of children are needed to demonstrate any benefit of probiotics in the management of *H. pylori* infection in children, including its effect on the severity of *H. pylori* gastritis. Additional work is necessary to determine the strain, dose and administration to be used. Long-term studies are also needed in children to prove whether the persistent suppressive effect of probiotics on *H. pylori* and its associated gastritis could prevent diseases such as gastric cancer or peptic ulcer.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (6): *Helicobacter pylori*

Role of *Helicobacter pylori* in gastric mucosa-associated lymphoid tissue lymphomas

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Abstract

Mucosa-associated lymphoid tissue (MALT) lymphoma is an indolent extranodal marginal zone B-cell lymphoma, originating in acquired MALT that is induced in mucosal barriers as part of a normal adaptive immune response to a chronic immunoinflammatory stimulus, most notably chronic infection by *Helicobacter pylori* (*H. pylori*). This antigenic stimulation initially leads to lymphoid hyperplasia; the acquisition of additional genetic aberrations culminates in the activation of intracellular survival pathways, with disease progression due to proliferation and resistance to apoptosis, and the emergence of a malignant clone. There are descriptions of MALT lymphomas affecting practically every organ and system, with a marked geographic variability partially attributable to the epidemiology of the underlying risk factors; nevertheless, the digestive system (and predominantly the stomach) is the most frequently involved location, reflecting the gastrointestinal tract's unique characteristics of contact with foreign antigens, high mucosal permeability, large extension and intrinsic lymphoid system. While early-stage gastric MALT lymphoma can frequently regress after the therapeutic reversal of the chronic immune stimulus through antibiotic eradication of *H. pylori* infection, the presence of immortalizing genetic abnormalities, of advanced disease or of eradication-refractoriness requires a more aggressive approach which is, presently, not consensual. The fact that MALT lymphomas are rare neoplasms, with a worldwide incidence of 1-1.5 cases per 10^5 population, per year, limits the ease of accrual of representative series of patients for robust clinical trials that could sustain informed evidence-based therapeutic decisions to optimize the quality of patient care.

Key words: Mucosa-associated lymphoid tissue lymphoma; Marginal zone lymphoma; *Helicobacter pylori*; Gastric lymphoma; Eradication therapy; Nuclear factor-kappa B pathway

Core tip: Mucosa-associated lymphoid tissue (MALT) lymphomas are indolent B-cell lymphomas, originating in acquired MALT induced as a response to a chronic immunoinflammatory stimulus, notably infection by *Helicobacter pylori* (*H. pylori*). Antigenic stimulation determines lymphoid hyperplasia; additional genetic aberrations activate survival pathways, with the emergence of a malignant clone. The digestive system (predominantly the stomach) is the most frequent location, reflecting contact with foreign antigens, mucosal permeability and intrinsic lymphoid system. Early-stage gastric MALT lymphoma can regress through the eradication of *H. pylori*. Immortalizing genetic abnormalities, advanced disease or eradication-refractoriness require treatment alternatives, presently not consensual. Representative clinical trials are needed to optimize patient care.

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INTRODUCTION

Extranodal marginal zone lymphomas of the mucosa-associated lymphoid tissue (MALT) type are indolent, low-grade, mature small B-cell non-Hodgkin lymphoid neoplasms (Table 1) that represent the paradigm for the association between tumorigenesis and a chronic inflammatory stimulus, and are one of the best models of the relationship between specific genetic events and oncogenesis[1-5].

Although rare, these neoplasms are clinically relevant due to their unique place in the oncology spectrum as a malignancy that, in many cases, can be cured with a short course of antibiotic therapy.

MALT LYMPHOMAS AND HELICOBACTER

Mucosa-associated lymphoid tissue

Primary lymphoid tissue can be found in the thymus and bone marrow, where lymphocytes differentiate from progenitor cells into functional, mature lymphoid cells. Secondary lymphoid tissue is present in the lymph nodes, in the spleen and in mucosa-associated lymphoid tissue; the latter, with numerous lymphocytes and antigen-presenting cells, develops in the stroma under the epithelium of mucosal barriers that are in contact with the outside environment (gastrointestinal, respiratory and genitourinary tracts), where antigens accumulate and are processed and presented to lymphocytes, as part of a normal adaptive immune response[6]. MALT, like the other components of the immune system, can give rise to a lymphoproliferative disease - the MALT lymphoma. The immune cell of origin of this malignant proliferation appears to be a marginal zone (post germinal center) B-cell present both in lymph nodes and in extranodal tissue, related to plasma cells[7-9].

Despite their association with mucosa-associated lymphoid tissue, MALT lymphomas rarely arise in native physiologic MALT; rather, the majority of cases develop on extranodal acquired MALT infiltrates induced by an immune response to a chronic antigenic stimulus[3,10]. The best studied causal associations are with chronic infections, with the highest levels of evidence being found for gastric MALT and *Helicobacter pylori* (*H. pylori*) gastroduodenitis[3,10].

Etiopathogenesis

Chronic antigenic stimulation and the microenvironment: These etiologic associations have led to the hypothesis that chronic or repeated immune stimulation leads to a lymphoid expansion which, in the presence of environmental and microenvironmental factors and a genetic predisposition, can culminate in the emergence of a malignant clone. The mechanisms underlying the antigen-dependence of MALT lymphomas, and the impact of the inflammatory microenvironment, have gradually been elucidated, with tumor progression now known to be driven by an interaction between B-cell receptor (BCR)-derived signals and T-helper (T_h) cell signals[11].

Table 1 World Health Organization-based classification of mucosa-associated lymphoid tissue lymphomas

Hierarchical classification
Tumors of hematopoietic and lymphoid tissue
Mature B-cell neoplasms
Non-Hodgkin (B-cell) lymphomas
Marginal zone (B-cell) lymphomas
Extranodal marginal zone lymphomas of mucosa-associated lymphoid tissue

Hierarchical classification of mucosa-associated lymphoid tissue lymphomas, according to the 2008 World Health Organization definitions. Adapted from Swerdlow *et al*[5], 2008.

It has been demonstrated that MALT lymphoma B-cells exhibit polyreactive surface BCR immunoglobulins, and that direct stimulation by the specific allo-antigens and auto-antigens recognized by these surface antibodies leads to the proliferation of tumor cells; after this oligoclonal expansion, a dominant lymphoma clone can surface through selective pressure[11-13]. BCR polyreactivity has been shown to include simultaneous intermediate affinity to self-antigens (including stomach extract) and foreign antigens (including Helicobacter sonicate), although some authors suggest that polyreactivity is exclusive of tumors carrying t(11;18), and that most other MALT lymphoma antibodies are monoreactive and of high-affinity[13,14].

It has also been shown that MALT lymphomas are infiltrated by type 2 T_h (T_h2)-polarized T-cells and that tumor proliferation is enhanced by intratumoral $CD4^+$ T-cells[11]. A large proportion of these $CD4^+$ T-cells are suppressive $CD25^+$ forkhead box P3 $(FOXP3)^+$ regulatory T-cells (T_{regs}), which are themselves recruited by tumor B-cells; higher numbers of tumor-infiltrating $FOXP3^+$ cells confer a better response to *H. pylori* eradiation therapy[11,15].

Bacteria-induced lymphomagenesis: *H. pylori* infection, generally acquired in childhood, is the most frequent chronic bacterial infection worldwide, and is a major cause of gastroduodenal disease, including chronic gastritis, benign peptic ulcers, gastric carcinoma and gastric MALT lymphoma, although only a very small proportion of *H. pylori*-infected subjects develop these complications[16-19]. In fact, in a population with an incidence of *H. pylori* infection of approximately 60%, only 24 cases of gastric MALT lymphoma were observed out of approximately 70000 gastroscopies performed over a period of 18 years[20,21]. The outcome of the infection depends on the host immune response mounted against *H. pylori*, es-

Table 2 Recurrent chromosomal translocations described in mucosa-associated lymphoid tissue lymphomas

Translocation	Fusion protein
t(11;18) (q21;q21)	API2-MALT1
t(1;14) (p22;q32)	BCL10-IGH
t(1;2) (p22;p12)	BCL10-IGK
t(14;18) (q32;q21)	IGH-MALT1
t(3;14) (p14;q32)	FOXP1-IGH

API2: Apoptosis inhibitor 2; MALT1: Mucosa-associated lymphoid tissue translocation protein 1; BCL10: B-cell chronic lymphocytic leukemia/lymphoma protein 10; IGH: Immunoglobulin heavy chain; IGK: Immunoglobulin *kappa* light chain; FOXP1: Forkhead box protein P1.

pecially the functionality of cytotoxic effector T-cells[16]. This has also been demonstrated for chronic atrophic autoimmune gastritis, secondary to the infiltration and destruction of the gastric mucosa by cytotoxic T-cells specific for *H. pylori* epitopes that cross-react with the gastric proton-pump[16].

Several arguments support the central role played by *H. pylori* in MALT lymphomagenesis. Chronic infection with *H. pylori* is significantly associated with the induction of gastric lymphoid follicles, representing the proposed first step in MALT lymphomagenesis of lymphoid expansion[20]. In addition, *H. pylori* infection can be demonstrated serologically in most patients, and the bacterium can be histologically identified in the gastric mucosa of the majority of gastric MALT lymphomas, with some series describing incidences as high as 92%, although the density and detectability of *H. pylori* decrease as the histology progresses from chronic gastritis to gastric MALT lymphoma[10,22-24]. These data suggest that bacterial colonization is important for early lymphomagenesis, but becomes less relevant as the disease progresses; in fact, a monoclonal B-cell clone can be identified in chronic gastritis, before the development of clinical lymphoma[24]. *In vivo* data in a murine model have shown that infection with *Helicobacter spp.* is able to reproduce most pathophysiological changes that take place during the early stages of MALT lymphomagenesis[11].

H. pylori eradication through specific antibiotherapy [classic triple therapy with amoxicillin, clarithromycin and a proton-pump inhibitor (PPI), or one of its variations] leads to lymphoma regression in 75% of cases, in a few weeks to 18 mo[10]. The odds of success associate with the clinical stage, being very high for early-stage lymphomas, lower for more advanced stages and practically nil once the serosa is breached. These observations also support the hypothesis that *H. pylori*-independence is a feature of lymphoma progression, associated with the acquisition of additional genetic alterations[10]. This aspect parallels the finding in gastric carcinoma (the intestinal type primarily associating with *H. pylori* infection) that the absence of active infection by *H. pylori* is a significant adverse prognostic factor, with one series finding a decrease in 10-year overall survival (OS) in locally advanced disease, from approximately 70% in *H. pylori*-positive patients to just over 20% in *H. pylori*-negativity[25].

The relationship between chronic infection with *H. pylori*, microenvironment and lymphomagenesis has been strengthened by the fact that tumor cells only proliferate in response to strain-specific *H. pylori* cell preparations when in the presence of tumor-infiltrating T-cells; on the other hand, the latter expand in response to *H. pylori* stimulation even when isolated from the tumor microenvironment[26]. The elimination of the stimulus to the T-cell expansion that sustains tumor-growth, through the eradication of *H. pylori*, leads to tumor regression[26]. The central role that tumor microenvironment T-cells play in MALT lymphomagenesis means that the modulation of local T-cell immunity could be an attractive therapeutic approach[27].

It has been suggested that lymphomagenesis and genetic aberrations are also facilitated by DNA-damage caused by reactive oxygen species produced by neutrophils as part of the immune response to an infection by *H. pylori* strains positive for the virulence factor cytotoxin-associated gene A (CagA)[10]. In fact, CagA-positive strains associate with higher grades of mucosal inflammation, severe atrophic gastritis and gastric carcinogenesis, and activate the phosphoinositide 3-kinase/AKT pathway, an anti-apoptotic, pro-proliferative survival pathway, contrary to CagA-negative strains[28,29].

Genetics of MALT lymphoma

Lymphomas present with several genetic aberrations, including translocations, point mutations, gene amplifications and deletions of genes (including tumor suppressors), some of which have been shown to have diagnostic and prognostic value. Non-random chromosomal translocations involving a limited group of genes are characteristic[30]. In MALT lymphomas, 5 recurrent cytogenetic alterations have been described, converging on the same intracellular pathways[31] (Table 2).

Genes and signaling pathways: The immunoglobulin (Ig) heavy chain gene (*IGH*) is frequently involved in translocations in MALT lymphomas and other lymphoproliferative diseases, as a consequence of the chronic antigenic stimulation which underlies the Etiopathogenesis of these neoplasms and the central role played by the BCR in lymphomagenesis[10]. The Ig *kappa* light chain (*IGK*) and *lambda* light chain genes can likewise be involved, through the same mechanism. In fact, B-lymphoid cells, as part of their normal immune response, undergo rearrangements of the Ig genes as part of somatic hypermutation and class-switch recombination[32]. These directed mutations originate a localized genetic instability that can lead to aberrant rearrangements, with the juxtaposition of oncogenes to Ig gene enhancers[32]. The continued enhancer activation as a normal response to immune stimulation will, in turn, result in the overexpression of the activated oncogene, with inflammation driving oncogenesis.

Normal lymphocyte function depends on the strict

regulation of the transcriptional activity of nuclear factor *kappa* B (NF-κB), and the deregulation of this signaling pathway is a contributor to lymphomagenesis[33]. NF-κB is a primary transcription factor normally sequestered in the cytoplasm[34]. As part of the innate immune response, it is a point of convergence of various pathways that originate on surface receptors, including the BCR, leading to inducible modifications of the expression of genes that modify the immune response, cell survival, proliferation and apoptosis[34]. Deregulation of pathways converging on NF-κB can thus lead to cellular immortalization, and is frequent in immune, autoimmune and oncologic diseases, including MALT lymphoma, where it is fundamental for the continued development of a lymphoma that has achieved *H. pylori*-independence[3,35].

Antigen stimulation of the BCR recruits B-cell chronic lymphocytic leukemia/lymphoma protein 10 (BCL10) to the antigen-receptor complex and this protein, in turn, links BCR signaling to the NF-κB pathway, through its interaction with mucosa-associated lymphoid tissue translocation protein 1 (MALT1)[10,36]. MALT1 oligomerizes with BCL10, leading to the downstream activation of NF-κB[10,34,37]. In the absence of oligomerization, wild-type MALT1 is incapable of activating NF-κB; however, when it oligomerizes in the absence of BCL10, it acquires the NF-κB-activating ability of the hetero-oligomer - MALT1 activity and NF-κB activation are thus dissociated from upstream signaling originating in the surface BCR and, consequently, from antigenic stimulation[10]. BCL10 also acquires the ability to constitutively activate NF-κB independently of antigenic stimulation, when it is overexpressed, such as when it is brought under the control of hyperactive promoter or enhancer regions, through chromosomal translocations[10,38]. None of these alterations, however, are independently sufficient for MALT lymphomagenesis, and the interaction with other immune, genetic and environmental factors is probably necessary for continued tumor growth[31].

Recurrent translocations: Rearrangements of the genes encoding the proteins described above - *MALT1*, *BCL10*, *IGH* and *IGK* - as well as *API2* and forkhead box P1 gene (*FOXP1*), result from the 5 recurrent translocations that have been described for MALT lymphoma (Table 2). The three most common and characteristic translocations - t(11;18) (q21;q21), t(1;14) (p22;q32) and t(14;18) (q32;q21) - are present with variable frequency depending on the tissue of origin of the lymphoma[10]. They generate oncogenic fusion proteins that activate the NF-κB pathway, and lymphomas with these translocations show an overexpression of NF-κB target genes[31,39].

The t(11;18) (q21;q21) results in the chimeric fusion of apoptosis inhibitor 2 (*API2*) and *MALT1*, originating a transcript that codes a functional fusion protein that has the ability of MALT1 to activate NF-κB but is controlled by the *API2* promoter, which is itself stimulated by NF-κB[10,38]. Therefore, this fusion gene results in a positive feedback cycle that leads to the unregulated, constitutive activation of NF-κB[34]. The API2-MALT1 fusion oncoprotein also contributes to the constitutive activation of NF-κB through an additional alternative non-canonical pathway[33,34]. Thus, this translocation is an important driver of MALT lymphomagenesis, immortalizing the cell and releasing it from BCR-antigen-dependence for its NF-κB activation and survival[10,40]. This is in agreement with the clinical observations that the presence of t(11;18) correlates with resistance to a successful eradication of *H. pylori*, that patients who respond to eradication therapy are generally negative for the fusion transcript, and that its presence is more frequent in *H. pylori*-negative than *H. pylori*-positive patients, suggesting that the latter need chronic stimulation of their BCR by antigen-antibody complexes for lymphoma cell survival[10,39-41]. The NF-κB-activating translocations t(1;14) and t(14;18) have similarly been noted to be associated with bacterial eradication-resistance[39].

Notably, t(11;18) is the most common structural chromosomal abnormality described in MALT lymphomas, being particularly frequent in gastric (reports ranging from 10%-35%), colonic and pulmonary locations[12,41,42]. It has a very high specificity (being exclusive or nearly-exclusive) for the MALT subtype, is the most specific of the recurrent translocations in these neoplasms, and is of high diagnostic value[43]. It is absent from non-complicated *H. pylori*-positive gastritis but often found in gastric MALT lymphoma patients infected with CagA-positive *H. pylori*[10]. The transcript is rarely present in MALT lymphomas with areas of high-grade (diffuse large B-cell lymphoma, DLBCL) transformation, leading some authors to consider it exclusive of low-grade cases[41,44]. On the other hand, it associates with advanced stages and submucosal involvement, being absent from lymphomas restricted to the mucosa[10,41].

The t(14;18) (q32;q21) results in the fusion of *IGH* with *MALT1*, inducing the overexpression of *MALT1*, which oligomerizes and activates NF-κB[10]. This translocation is virtually absent from gastric locations, but variably common in different extragastric tumors, with reports ranging from 20% of salivary gland to 100% of hepatic tumors[45-47].

The t(1;14) (p22;q32) induces the juxtaposition of *BCL10* with the *IGH* gene enhancer region, with a resulting overexpression of BCL10 and activation of NF-κB[38]. In the t(1;2) (p22;p12) variant, *BCL10* is juxtaposed to *IGK*, originating an identical overexpression of BCL10. The two variants, though characteristic of MALT lymphomas, are found in under 4% of described cases and associate frequently with other cytogenetic aberrations, such as trisomy 3[10].

The t(3;14) (p14;q32) apposes the *FOXP1* with the *IGH* enhancer, resulting in the overexpression of the former[48]. FOX family proteins have been shown to be involved in signal transduction that mediates proliferation, differentiation and the immune response[49]. Though its precise mechanism of action remains to be clarified, in MALT lymphoma (as in DLBCL) *FOXP1* overexpression

has been described as an adverse prognostic factor[48-51]. Like t(14;18), its frequency varies among different anatomical locations, with the original series describing incidences ranging from 0% in gastric locations to 50% of thyroid samples[48].

Other somatic alterations: Apart from the characteristic translocations, several other somatic genetic alterations can be identified in MALT lymphomas, including numeric chromosome aberrations and allelic imbalances. The specific frequencies of each genetic aberration vary in the literature, with reports addressing distinct lymphoma locations and stages, using differing methodologies and focusing on series from separate geographical locales. It has been suggested that these geographical differences reflect a true heterogeneity in the distribution of genetic aberrations, and not just different sampling methods, and that the different anatomical locations are a reflection of distinct processes of lymphomagenesis[44,52].

Epidemiology

MALT lymphomas represent approximately 7% of newly-diagnosed lymphomas[8]. They are a rare malignancy, with a worldwide incidence estimated at 1-1.5 cases per 10^5, per year[5]. Gastric cancer, in comparison, is 5 to 10-fold more frequent (United States National Cancer Institute Surveillance Epidemiology and End Results data). As with other indolent lymphomas, the incidence increases with age, with the majority of patients being over 50 years old (with a median of 61)[5].

These lymphomas can affect practically all organs and systems, although different anatomical locations have a large geographic variability, which has been partially attributed to a distinct epidemiological risk factor distribution[53]. Overall, the digestive system is the most frequently involved location, reflecting the gastrointestinal tract's unique characteristics of contact with foreign antigens, mucosal permeability, large extension and intrinsic lymphoid system[10,45,54]. In fact, MALT lymphomas represent a large proportion of all gastrointestinal lymphomas: in a revision of B-cell gastrointestinal lymphomas, one-fifth were pure MALT lymphomas and a further 8% were MALT lymphomas with a DLBCL component[55]. Gastrointestinal involvement by B-cell lymphomas is most common in the stomach (which accounts for 60%-75% of gastrointestinal lymphomas, and for over 50% of all MALT locations), followed by the small intestine, colon and rectum[3,54,56-58].

The involvement of various non-contiguous sites (including both different systems and discrete segments of the same system, such as different aspects of the gastrointestinal tract, separated by healthy tissue) is common in MALT lymphomas, both at diagnosis and throughout the evolution of the disease, and has been interpreted as recurrence, dissemination or independent synchronous or metachronous development[12,59,60]. There are descriptions of the concomitance of MALT lymphoma with other lymphomas and even with other malignancies, such as the coexistence of primary gastric MALT lymphoma and Epstein Barr virus-associated gastric carcinoma, or of colonic adenocarcinoma and gastric MALT[61-63]. It has been proposed that, in these circumstances, treatment decisions should prioritize the tumor with the worst prognosis at the moment of diagnosis, which is generally the carcinoma[64].

Diagnosis

The diagnosis of MALT lymphoma rests on the clinical suspicion of a lymphoproliferative disease or another malignancy, confirmed by histopathologic data; the latter must be complemented by the judicious use of immunohistochemistry (and eventually flow cytometry), cytogenetics and molecular biology, moreover considering that the histological differential diagnosis between severe gastritis and early stage lymphoma can be difficult[4].

Histopathology: The histopathologic evaluation of a tissue biopsy sample remains fundamental for the diagnosis of MALT lymphoma. This lymphoma is characterized by the presence of a typical infiltrate located in the marginal zone of follicles with reactive germinal centers, with possible extension into the interfollicular region, made up of small, morphologically heterogeneous monoclonal B-cells, originating in post-germinative memory cells, and including centrocyte-like marginal zone cells, monocytoid B-cells, immunoblastic and centroblast-like cells; plasmocytes can be seen in the sub-epithelial zones and are monoclonal in up to half of cases[6,10,18,45]. Pathologic acquired MALT and MALT lymphoma are similar to physiological MALT[18]. Therefore, the principal diagnostic criterion for MALT lymphoma is the invasion and destruction of the adjacent epithelium, originating typical lymphoepithelial lesions, as described by Wotherspoon, although the European Society for Medical Oncology (ESMO) has recently determined by consensus that the presence of these lesions is neither essential for, nor specific of, a diagnosis[10,18,22,65]. Immunohistochemistry can be a valuable aid in the differentiation between MALT lymphomas and other small cell lymphomas, including follicular lymphoma, chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) and even mantle-cell lymphoma (MCL)[18,45] (Table 3). Immunophenotyping can also contribute to the differential diagnosis between small cell lymphomas[45]. MALT lymphoma B-cells have an immunophenotype that is identical to the normal phenotype of a non-neoplastic marginal zone lymphocyte, with positivity for the B-cell surface markers CD19, CD20 and CD22 and negativity for CD5 (unlike CLL/SLL), cyclin D1 (unlike MCL) and CD10[18,45,65]. Malignant cells exhibit light-chain restriction, as a marker of clonality[66]. It has been further suggested that the microRNA signature can be informative in the distinction between gastritis and MALT lymphoma[67].

Although MALT lymphoma is a low-grade disease, with large transformed cells being rare in the neoplastic infiltrate, it can undergo transformation to an aggressive

Table 3 Useful phenotypic markers for the differential diagnosis of mucosa-associated lymphoid tissue lymphoma

Antigen	Notes
sIg^+	B-cell receptor
$CD19^+$	Pan-B-cell marker
$CD20^+$	Pan-B-cell marker
$CD22^+$	Pan-B-cell marker
$CD79a^+$	Pan-B-cell marker
$CD5^-$	Positive in CLL/SLL
$CD10^-$	Positive in follicular lymphoma
$CD23^-$	Positive in CLL/SLL
Cyclin $D1^-$	Positive in mantle cell lymphoma

CD: Cluster of differentiation; sIg: Surface immunoglobulin; CLL/SLL: Chronic lymphocytic leukemia/small lymphocytic lymphoma.

diffuse large B-cell lymphoma (the most common histological type of primary gastric lymphoma, representing over half of cases), through poorly understood mechanisms[45,51,68]. Transformed MALT/DLBCL appears to have a similar prognosis to *de novo* DLBCL, with overlapping progression-free and overall survivals[69].

Cytogenetics and molecular biology: The identification of the characteristic recurrent chromosomal translocations, by conventional cytogenetics, FISH or molecular biology, is informative and can contribute to the differential diagnosis of MALT lymphoma, as described above.

Medical image: Imaging studies are fundamental not only for the diagnosis but also for the adequate staging of the lymphoma at presentation.

Esophagogastroduodenoscopy with multiple biopsies is the gold standard for the diagnosis of gastric MALT lymphoma. In a 24-patient series, the most common endoscopic findings were mild hyperemia (67%), superficial erosions (17%) and superficial ulcers (17%)[21]. Gastric ulcers, especially when unresponsive to conventional treatment, should be biopsied due to the risk of malignancy; although gastric carcinoma is the usual finding in malignant gastric ulcers, some cases of gastric lymphoma can also present as ulcers, including with local complications, such as perforation[70]. Push enteroscopy with serial biopsies is safe and easy, and can detect the synchronous involvement of the duodenum and jejunum by MALT lymphoma, a finding that was present in 11% of patients in a retrospective series[59]. Colonoscopy is also able to identify macroscopic changes in the mucosa (such as discoloration with a reduction of superficial vessels) in colorectal MALT lymphoma and, according to some authors, should also be part of the diagnostic workup of gastric MALT lymphoma to screen for metachronous involvement[71]. Serial esophagogastroduodenoscopies with multiple biopsies are mandatory for the follow-up of post-remission gastric MALT lymphoma, especially in early-stage disease, where recurrence tends to be localized to the mucosa and undetectable by other imaging modalities[72].

It has been suggested that the use of magnified endoscopy techniques for the evaluation of the microstructural pattern of the lesion and distribution of abnormal vessels could be useful both for diagnosis and follow-up[73]. In a series of patients with localized gastric disease, nonstructural areas with abnormal vessels were present at magnified esophagogastroduodenoscopy in all patients at diagnosis, disappearing with histopathologic remission[73]. Compared to histopathology, nonstructural areas had a sensitivity of 77% and a specificity of 87%, while the presence of abnormal vessels had both a sensitivity and specificity of 86%[73].

Table 4 Association between abdominal computerized tomography findings and the likelihood of low-grade and high-grade lesions

Low-grade lesions	High-grade lesions
Normal scans	Abnormal scans
Gastric wall thickening < 5-10 mm	Diffuse gastric wall thickening > 10 mm
Small depressed lesions with vague margins	Well-demarcated masses with homogeneous attenuation and mild contrast enhancement
	Perigastric adenopathies more likely

Adapted from Hayashi *et al*[76], 2010.

Endo Ultrasonography enables the endoscopist to evaluate the degree of organ involvement and infiltration of contiguous structures in a single procedure, which is fundamental for staging[10]. The presence of diffuse parietal thickening in endo-ultrasonography is suggestive of infiltration by lymphoma[71]. The ultrasonographic appearance of MALT lymphoma can be characteristic in some locations, and it has been suggested that when sonographic findings are characteristic, excisional biopsy can be replaced by ultrasound-guided core-needle biopsy[74].

Abdominal computerized tomography (CT) can detect locally advanced gastric MALT lymphoma, presenting as a diffuse or localized parietal thickening, as well as lymphadenopathy, local complications (including perforation) and hepatosplenomegaly[75]. Gastrointestinal dissemination can manifest as circumferential parietal thickening of an intestinal segment, or localized polypoid masses with homogeneous and isoattenuating or hypoattenuating enhancement[71,75]. Three-dimensional reconstruction in gastrointestinal lymphomas correlates with the underlying histopathology, with an increased likelihood of low-grade gastric MALT lymphoma in patients with normal scans, with minimal gastric wall thickening (5-10 mm) or with small depressed lesions with vague margins[76] (Table 4). On the other hand, a severe diffuse thickening of the gastric wall (> 10 mm), focal well-demarcated masses, or masses with homogeneous attenuation and mild contrast enhancement, suggest high-grade lesions; perigastric adenopathies are also more likely in the latter than in low-grade lymphoma[76]. In contrast, multiple lymphomatous polyposis is common in MCL, a bulky mass with uniform isoattenuating in the right lower quadrant suggests Burkitt lymphoma, and thickened nodular folds with multiple ulcerative lesions, perforations and obstruction are typi-

Table 5 Comparison of four frequently used staging systems for primary gastrointestinal lymphomas

Tissue invasion	Ann arbor	Radaszkiewicz	Lugano	Paris
Gastrointestinal tract	I E	I E	I	T^1 N0 M0
Mucosa or submucosa	I E	I E1	I	T1 N0 M0
Mucosa	I E	I E1	I	T1m N0 M0
Submucosa	I E	I E1	I	T1sm N0 M0
Muscularis propria or subserosa	I E	I E2	I	T2 N0 M0
Serosa	I E	I E2	I	T3 N0 M0
Intra-abdominal extension			II	
Adjacent tissues or organs	I E	I E	II E	T4 N0 M0
Regional lymph nodes	II E	II E	II 1	T^1 N1 M0
Infradiaphragmatic distal lymph nodes	II E	II E	II 2	T^1 N2 M0
Disseminated disease			IV	
Supradiaphragmatic lymph nodes	III E	III E	IV	T^1 N3 M0
Non-contiguous gastrointestinal[2]	IV E	IV E	IV	T^1 N^1 M1
Non-contiguous metastasis[3]	IV E	IV E	IV	T^1 N^1 M2
Marrow involvement	IV E	IV E	IV E	T^1 N^1 M^1B1

In the case of synchronous lesions originating in the gastrointestinal tract, staging refers to the characteristics of the most advanced lesion. Note that the Lugano system does not include a stage III. [1]Any subtype of tumor extension (T1 to T4) or nodal (N0 to N3) or metastatic (M0 to M2) involvement; [2]Non-contiguous gastrointestinal involvement refers to the presence of lymphoma in more than one gastrointestinal site with segments of discontinuity that are free of disease (such as the involvement of the stomach and rectum, with a free small intestine and bowel); [3]Including the non-contiguous involvement of the peritoneum. References for the 4 staging systems are given in the main text.

cal of T-cell enteropathy; DLBCL is multiform and often invasive[76]. Despite its utility for initial staging, abdominal CT scanning does not appear to be useful for the follow-up of localized gastric MALT lymphoma[72]. In a series of patients with early-stage gastric MALT lymphoma in complete remission, 5.7% had recurrent disease, which was confined to the mucosa and, therefore, undetectable on CT[72].

Staging

The staging of both Hodgkin and non-Hodgkin lymphoma is standardized through the Ann Arbor system, with the Costwolds modifications[77,78]. Due to the intrinsic limitations of this system in primary extranodal lymphomas of the gastrointestinal tract, the Radaszkiewicz and the Lugano modifications were proposed, with variable success[79,80]. To overcome the perceived shortcomings of the various lymphoma staging system adaptations, both for the correct definition of the primary tumor extension and depth of infiltration, and as a basis for therapeutic decisions, the European Gastro-Intestinal Lymphoma Study Group proposed the Paris System [tumor-node-metastasis (TNM)-B], an adaptation of the existing TNM system in mainstream use for the classification of non-hematologic solid malignancies, for the staging of primary gastrointestinal lymphomas[81] (Tables 5 and 6).

Table 6 Tumor-node-metastasis staging system qualifiers

	Stage	Definition
Tumor	Tx	Extension of lymphoma not established
	T0	No evidence of primary lymphoma
Nodes	Nx	Nodal involvement not evaluated
	N0	No evidence of nodal involvement
Metastasis	Mx	Dissemination of lymphoma not evaluated
	M0	No evidence of lymphoma dissemination
Bone marrow	Bx	Bone marrow infiltration not evaluated
	B0	No evidence of bone marrow infiltration

Table 7 International prognostic index for aggressive B-cell lymphomas

Prognostic factor	Quantification
Advanced age	> 60 yr
Advanced stage	Ann arbor stages III or IV
High tumor burden and activity	Increased serum lactate dehydrogenase
Poor performance status	ECOG ≥ 2
Multifocal distribution	Two or more extranodal sites

The International Prognostic Index is calculated by adding 1 point for each adverse risk factor. ECOG: Eastern Cooperative Oncology Group performance status scale.

Prognosis

Most MALT lymphomas are at diagnosis characterized by non-disseminated (early-stage) disease, with both marrow and distal nodal involvement being rare, although regional lymph node infiltration is relatively frequent in gastric MALT lymphomas[45]. Staging alone is not sufficiently predictive of disease outcome in lymphoproliferative diseases, with survival being influenced by several concurrent prognostic factors. To adequately integrate all these factors into the clinical decision, prognosis can be quantified in B-cell lymphomas through internationally validated scales or indices, such as the International Prognostic Index (IPI), which was developed for aggressive B-cell lymphomas[82] (Table 7). There is no prognostic index that is specific for MALT lymphomas. However, it has been demonstrated that IPI scores correlate significantly with time to relapse in MALT lymphomas, differentiating low, low-intermediate and high risk groups; on the other hand, data regarding the utility of the FLIPI (follicular lymphoma IPI) in this group of patients is contradictory[83,84]. Additional indicators of poor prognosis include the presence of a large-cell component at diagnosis, B symptoms (unexplained fever, night sweats and unintentional weight loss), high serum β2-microglobulin or serum lactate dehydrogenase, low serum albumin, bone marrow failure (evidenced by anemia or thrombocytopenia), advanced

age (over 60 years) or poor performance status (2 or above on the WHO/Eastern Cooperative Oncology Group scale), and the presence of a bulky tumor[82,85,86]. The presence of extragastric disease also appears to have prognostic value[83]. The absence of complete remission with first-line treatment is a further *a posteriori* indicator of poor prognosis. We have discussed above how genetic aberrations, such as the presence of the t(11;18), correlate with resistance to treatment. Rearrangements of the *BCL6* locus, or BCL6 protein overexpression, appear to associate with large-cell transformation of MALT; OS in *de novo* and MALT-transformed gastric DLBCL correlates strongly with BCL6 overexpression[87,88].

Treatment

Current guidelines are consensual in indicating *H. pylori* eradication therapy as the first line approach in gastric MALT lymphoma[89]. However, due to the paucity of extensive series of patients with MALT lymphomas and, more importantly, of prospective clinical studies, the optimal treatment of *H. pylori*-negative and eradication-resistant *H. pylori*-positive gastric lymphomas has not been convincingly established[65,89]. Therefore, different centers report a variety of approaches, many of which have relevant side-effects[8,57,90].

These lymphomas follow an indolent clinical course with prolonged OS (80% at 5 years) and disease-free survival, on par with other low-grade lymphomas and, in early stage disease, tend to respond to a wide variety of treatment approaches; however they are characterized by a high recurrence rate, with most patients relapsing within 5 years, often in organs with acquired MALT that are distant from the original location[45,90-92]. Second remissions can be regained with retreatment; however, the disease-free interval tends to decrease after each subsequent remission[91]. Early-stage disease tends to remain localized for a long time, and responds satisfactory to local treatment approaches, such as surgery or radiotherapy[90]. However, survival correlates inversely with the stage at diagnosis (90%-95% at 5 years for stage Ⅰ, 75% for stage Ⅱ and as low as 30% for stage Ⅳ), with about one-third of patients presenting with advanced disseminated disease at diagnosis and requiring systemic treatment[90].

Antibiotherapy: Since MALT lymphomas are indolent neoplasms, in selected patients with asymptomatic or minimally-symptomatic non-gastric MALT lymphoma without a large-cell component, a strategy of expectant active surveillance of the patient (watchful waiting) with repeated imaging studies and hematological monitoring can be the most adequate approach at diagnosis, moreover due to the possibility of spontaneous regression of MALT lymphomas, which can occur even when there is unequivocal histological confirmation of the lesion and when there is a transformed high-grade component[93,94].

On the other hand, the antibiotic eradication of *H. pylori* infection is the first line treatment for gastric MALT lymphomas in Ann Arbor Stage Ⅰ E (representing the majority of tumors at diagnosis), leading to a complete endoscopic and histopathologic remission with an excellent prognosis and the possibility of cure in approximately 80% of patients (most patients in Stage Ⅰ E1 and a smaller proportion of patients in stage Ⅰ E2), while lymphomas in Stage Ⅱ E and above are usually less responsive; regression of Stage Ⅰ E (and some Ⅱ E) gastric DLBCL, both *de novo* and transformed from MALT, following *H. pylori* eradication therapy, has also been described[10,95-99]. Although the probability of MALT lymphoma regression in response to a successful *H. pylori* eradication is influenced by the patient's cytogenetics, through the mechanisms described above, it has been suggested that introducing empiric eradication therapy in the absence of molecular testing is clinically justified, due to the high remission rates that can be achieved[4]. Nevertheless, when these methods are available, testing for t(11;18) could be a helpful *a priori* predictor of response to therapy[65,100].

Testing for *H. pylori* infection should be performed in all patients with gastric MALT lymphoma, through esophagogastroduodenoscopy with biopsy (for histopathology, culture or a rapid urease test); through a fecal antigen test; or through a urea breath test[101]. Comparing the available tests for *H. pylori*, the antigen test has been noted to have a higher sensitivity and negative predictive value (both 100%) than the rapid urease test, while the latter was found to have a higher specificity and positive predictive value[102]. PPI should be suspended at least one week before testing[101]. Irrespective of the test results, the ESMO consensus is that "eradication therapy must be given to all gastric MALT lymphomas, independently of stage or histological grade"[65].

The first line therapy for eradication is the triple association between a PPI, clarithromycin and either metronidazole or amoxicillin[65,101]. In special select cases, the antibiotics may have to be selected through an antibiotic sensitivity test, as in the case of known antibiotic allergies[103]. Successful eradication should be confirmed by repeat testing for *H. pylori* four weeks or more after completion of therapy, due to potential treatment failures, which are partly due to compliance issues[101]. We have described a failed eradication rate of nearly 20% with an amoxicillin/clarithromycin/PPI association, in a population of unselected *H. pylori*-positive patients without gastric lymphoma, despite *ex vivo* sensitivity of all strains to the two antibiotics used[104]. In the case of treatment failure, eradication should be re-attempted with a quadruple association of a PPI, tetracycline, metronidazole and a bismuth salt[101].

While over 80% of patients can achieve a complete lymphoma remission (according to GELA criteria[105]) with successful *H. pylori* eradication, there are no clear predictive factors for lymphoma response to eradication, and primary refractoriness can be found in 10%-20% of low-grade gastric MALT lymphomas[97,106]. In a series of Ann Arbor Stage Ⅰ E1 patients, there were 7% of non-responders, who were not different from responders in gender, age, endoscopic appearance or large-cell

Table 8 Chemotherapy treatment options in American and European guidelines

Drug group	Drugs
Alkylators	Chlorambucil[1]
	Cyclophosphamide[1,2]
	Bendamustine[1,2]
Purine nucleoside analogues	Fludarabine[1,2]
	Cladribine[1]
Anthracyclines and anthracenediones	Doxorubicin[2]
	Mitoxantrone[2]
Vinca alkaloids	Vincristine[2]

Reflecting the lack of clear consensus, current guidelines propose different associations of these agents as monotherapy, combination chemotherapy, and combined immunochemotherapy with rituximab. [1]European Society for Medical Oncology 2013 guidelines; [2]National Comprehensive Cancer Network 2011 guidelines.

component size; significantly, complete remissions were achieved in over 98% of distal tumors, but only in 70% of proximal tumors[99].

The fact that gastric MALT lymphoma regression, in response to *H. pylori* eradication, can take up to 18 mo, means that refractoriness should not be assumed prematurely, and determines a compulsory extended follow-up period, with regular esophagogastroduodenoscopies and repeat biopsies to demonstrate complete remission, although the optimal frequency of endoscopic evaluation has not been definitely established[4,10]. A period of watchful waiting with repeated esophagogastroduodenoscopic biopsies has also been proposed as a valid option after successful gastric MALT lymphoma regression following eradication[107]. The fact that 5% of patients with gastric MALT lymphoma in complete remission develop local metachronous gastric carcinoma despite *H. pylori* eradication (an incidence reported to be 6 to 9-fold that of the general population), diagnosed by long-term endoscopic follow-up, also underlines the importance of close endoscopic surveillance[106,108]. Nevertheless, the ideal duration of the follow-up period after initial eradication treatment remains to be defined, with some authors suggesting immediate treatment after a successful eradication without lymphoma remission, while others propose continued watchful waiting[97]. The identification of resistance-associated genetic aberrations, such as t(11;18), could be an indication of true refractoriness to eradication, guiding therapeutic decisions. Likewise, the presence of a large-cell component should help inform a choice to opt for alternative therapies if eradication fails to induce regression. The presence of symptoms that interfere with the patient's quality of life is an indication to suspend watchful waiting and introduce treatment[109].

Complete remissions that are achieved through *H. pylori* eradication are prolonged. In a series of stage I E1 patients in complete remission, after a median of 35 mo of follow-up only 5% showed lymphoma recurrence, which was limited to the mucosa and only detectable on endoscopic biopsies[72]. In 57% of these patients, recurrence was associated with re-infection with *H. pylori*, and regressed after re-eradication; the tumors in the remaining patients were *H. pylori*-negative and regressed spontaneously[72]. The presence of persistent minimal histological residuals after *H. pylori* eradication with endoscopic normalization can be managed through a watchful waiting approach with regular endoscopic biopsies, with over 95% of patients either maintaining stable minimal histological residuals or eventually achieving a complete response, as was demonstrated by two series of over 100 patients[98,107,108].

Although there have also been descriptions in small series of regression of *H. pylori*-negative MALT lymphomas after eradication therapy, which have been interpreted by the authors as being causally related, the physiopathologic basis for this finding needs to be further explained[110,111].

Local treatment approaches: In gastric MALT lymphoma, the current view is towards stomach-conserving conservative treatment, avoiding first-line surgical resection[98]. Nevertheless, a surgical approach can be curative in MALT lymphomas, especially when the lymphoma is an unexpected finding after resection, or collocates with a more aggressive carcinoma that is completely resected[61,112,113]. Regardless of curative intent, an invasive approach can be indicated in the first line for the control of local complications of the tumor.

Radiotherapy has a high curative potential in the stomach-conserving treatment of gastric MALT lymphoma, in *H. pylori*-negative patients or following a lack of response to eradication in *H. pylori*-positive cases, with 80% of eradication-refractory patients achieving a complete remission with radiotherapy; a dose of 30-40 Gy in 15-20 fractions has been proposed[58,98,106,114].

Chemotherapy: Eradication-refractory gastric MALT lymphomas have high rates of response to chemotherapy, and this is a valid approach after confirmed failure of first-line eradication. Likewise, it is justified in systemic disease with *a priori* dissemination. In contrast, the use of chemotherapy in localized MALT lymphomas after a successful response to *H. pylori* eradication, proposed by some authors to prevent recurrence, is still controversial, with no evidence to support it[4,8,115].

Several chemotherapeutic drugs have been assayed when systemic therapy is warranted, both as single-agent treatments and in combinations, in case-series reports or in small-sized clinical trials, with varying results, depending on the anatomical location and the stage of the lymphoma (Table 8). Advances have been scarce in the 6 years since the extensive treatment review by Morgner and colleagues; as of 2013, there is no definitive data to support the choice of one modality of systemic treatment over the others, and the benefit of chemotherapy over local treatment approaches in earlier stage disease is still not clear[65,116]. A controlled prospective clinical trial of early-stage (I E and II E) gastric MALT lymphoma comparing surgery, radiotherapy, and chemotherapy with

cyclophosphamide, vincristine and prednisolone, with or without doxorubicin (CVP/CHOP), showed a significantly higher event-free survival with combination chemotherapy, compared to either surgery or radiotherapy, but with identical OS in all three arms[117].

It has been noted that tumor microenvironment T-cells play an important role in lymphomagenesis induced by chronic antigenic stimulation, underlying the potential utility of chemotherapeutic agents that simultaneously target malignant B-cells and microenvironment T-cells, such as single-agent nucleoside analogues[27]. Fludarabine demonstrated a significant reduction in peripheral blood T-cells, compared to eradication alone, while in biopsy samples there was an increase in T_{regs}[27]. Cladribine achieved a complete response rate of 100% in primary gastric lymphomas, with an overall (gastric and extragastric) survival at 80 mo of 84%[118]. On the other hand, the T-cell modulation associated with nucleoside analogues such as fludarabine and cladribine can lead to long-term immunosuppression and increased infectious risk, with increased morbidity[90]. A phase Ⅱ trial of gemcitabine, a less T-suppressive analogue expected to overcome this problem, was discontinued due to disappointing results[90].

Single-agent alkylators, including chlorambucil and bendamustine, have shown clinical effectiveness with acceptable toxicity, demonstrating that this is another valid treatment approach[91,115,119,120]. However, it has been suggested that t(11;18) is a marker of non-response to alkylating regimens[65].

Several of the newer agents have also been tried in gastric MALT lymphomas. Thalidomide is an antiangiogenic and immunomodulatory drug with anti-NF-κB activity, which justifies its potential utility in MALT lymphomas[35]. It has been used as a salvage therapy in a series of *H. pylori* eradication-refractory chemo-resistant gastric MALT lymphomas, with an overall response rate (ORR) of 0% in patients with the API2-MALT1 transcript and of 86% in patients without the transcript; the latter (but not the former) showed a significant downregulation of the expression of NF-κB in residual neoplastic cells and tumor microenvironment[35]. These data suggest that the presence of t(11;18) is also predictive of non-response to thalidomide[35].

The role of the ubiquitin proteasome system (UPS) in the regulation of the NF-κB pathway serves as the rationale behind the use of proteasome inhibitors in the treatment of MALT lymphomas[121]. Previous basic and clinical experience with these agents has demonstrated that they disrupt multiple UPS-dependent cellular pathways, with apoptosis as the final event[8]. However, trials of bortezomib have demonstrated high rates of toxicity at full-dose, which persisted with a reduced-dose protocol that had an ORR under 50%[8,121]. Given the indolence and prolonged survival of MALT lymphoma, acute and long-term toxicity should be taken into account when interpreting results and comparing risk-benefit ratios of different treatment approaches[8].

Monotherapy with an anti-CD20 monoclonal antibody (rituximab) can induce sustained complete remissions of MALT lymphoma, with descriptions of success in both localized and systemic disease, and with retreatment at relapse leading to reinduction of complete remission[122-125]. In MALT lymphoma, as in other B-cell lymphomas, rituximab has also been used as part of combination immuno-chemotherapy and radiotherapy, with good results, improving the responses to single-agent or multiple-agent chemotherapy, with tolerable side-effects[71,91,126-128]. However, some trials suggest that time to progression was not improved, while others fail to find an improvement with rituximab, results that highlight the importance of starting well-designed phase Ⅲ studies that can clarify the role of the various treatment approaches and of combination modalities[7,129].

An alternative to rituximab is radio-immunotherapy with ^{90}Y-ibritumomab tiuxetan, an anti-CD20 monoclonal antibody containing a radioactive isotope that was able to induce high rates of complete remissions of up to 24 mo in highly treated refractory patients, while permitting a ten-fold reduction in the dose radiotherapy, potentially overcoming some of the local complications of the latter[130,131].

CONCLUSION

MALT lymphomas are rare and heterogeneous malignancies that occupy a unique position in the spectrum of oncologic disease, as they can potentially be cured with a simple course of antibiotics.

Nevertheless, as indolent lymphomas, they present to the clinician the singular challenge of having to identify the optimum balance between effective therapy and minimal toxicity for a neoplastic disease that can have a decades-long course of remission and relapse, often in the absence of robust data and representative series on which to sustain an evidence-based practice of medicine.

The known association of gastric MALT lymphoma with chronic immune stimulation through *H. pylori* infection has offered invaluable insights into lymphomagenesis and, by extension, the mechanisms of neoplastic transformation in general. The knowledge thus acquired has, in turn, exposed key molecules of cell-cycle regulation, survival, apoptosis and proliferation, which can be manipulated as specific therapy targets. Such findings can often be reciprocally translated between MALT lymphomas and other lymphoproliferative and plasma cell diseases, which share common pathways of malignization.

The clinical translation of these findings must, necessarily, rely on strengthened long-term multicentric international collaborations to enable the accrual of representative numbers of patients for epidemiologic studies and prospective, randomized, blinded clinical trials. Only then can we hope to move towards the truly targeted, personalized treatment approach that these patients require.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (6): *Helicobacter pylori*

Helicobacter pylori: Future perspectives in therapy reflecting three decades of experience

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Abstract

The rising prevalence of antibiotic resistance has created a need to reassess the established *Helicobacter pylori* (*H. pylori*) eradication protocols, and to develop new ones. Various bacterial and host factors are evaluated, and their contribution to eradication failure is estimated. For a long time being considered the cornerstone eradication scheme, the standard triple therapy has been replaced with novel, more efficient regimens, namely sequential and concomitant, along with the emergence of a new design of bismuth quadruple therapy. A rescue levofloxacin based regimen has overcome the fear of therapy failure due to higher prevalence of dual resistant (clarithromycin and metronidazole) *H. pylori*. Culture-free and efficient susceptibility test are reestablishing the concept of tailored therapy, making eradication success close to originally desirable rates. Alleviating therapy side effects and improving patient compliance are as important as choosing appropriate eradication schemes, so various probiotic compound supplements are taken into consideration. Finally, we summarize the emerging efforts and obstacles in creating efficient *H. pylori* vaccine.

Key words: *Helicobacter pylori*; Eradication therapy; Antibiotic resistance; First line therapy; Rescue therapy; Sequential therapy; Bismuth-containing quadruple therapy; Concomitant quadruple therapy; Hybrid (dual-concomitant) therapy

Core tip: In this article the authors have made a review of the most important literature with knowledge of various factors affecting *Helicobacter pylori* eradication success. The paper presents an analysis of established and new eradication regimens, as well as factors affecting their performance. Since the last 3 decades many new developments appeared in the field of this intriguing infection, along with implementation of recently published guidelines. Authors made a new look to future perspectives in managing this complex infection.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a gram-negative, microaerophilic bacterium inhabiting human stomach, first isolated by Warren and Marshall in 1983, and that has since then become a point of interest worldwide[1]. It is a major pathogen causing gastric and duodenal ulcer, gastric cancer and mucosa-associated lymphoid tissue

lymphoma[2,3]. Recently, it has been linked to extragastric malignancies such as colorectal adenocarcinoma and nonmalignant diseases such as iron deficiency anemia, idiopathic thrombocytopenic purpura and vitamin B_{12} deficiency, with new data on its role in neurodegenerative diseases and metabolic syndrome[2,4,5].

During three decades of *H. pylori* eradication, various therapeutic protocols have emerged, with the standard triple therapy recently succeeding generally bellow 80%, far away from the originally expected rate of > 90%[6-9]. Possible factors causing progressive eradication therapy failure have been evaluated, with the consensus that antibiotic resistance and patient compliance are the most important ones[6-9]. Accordingly, common opinion is that local antibiotic resistance screening and detailed evaluation of patient prior antibiotic usage, are main steps in preventing further eradication rate decline[2,7,9,10-12].

In this article we will review the most important results of various established and novel eradication protocols and the factors affecting them, along with a look to new perspectives in managing this complex and intriguing infection.

BACTERIA AND HOST INFLUENCING ERADICATION

There are multiple factors, concerning both the bacteria and the host, making *H. pylori* eradication so difficult. Factors affecting proton pump inhibitor metabolism and bioavailability, such as CYP2C19 and MDR1 polymorphism, and the IL-1B polymorphism affecting intragastric acidity, were implied in eradication success rate, with fast metabolizers and acid hypersecretory found to have lower therapy success[6,13,14]. Several studies have shown lower eradication rates in patients with non-ulcer dyspepsia compared to peptic ulcer disease. The reason may be that patients with non-ulcer dyspepsia seem to be infected with less virulent, slow-proliferating strains, making them less susceptible toward antibiotics, with those strains more frequently being resistant to clarithromycin[13,14]. More contradictory data were published about negative impact of increased body mass, diabetes and smoking on eradication rate[13,15]. Finally, before evaluating any cause of eradication failure, physicians must be aware of patient compliance, as those taking < 80% of their treatment regimen have a high chance of failure and subsequent antimicrobial resistance[12,14].

Multiple bacterial factors are influencing eradication therapy success rate, with the development of resistance to antibiotics as the most important[9,10,16]. Recent multicenter study by Megraud *et al*[16] showed that resistance rates in Europe for adults were 17.5% for clarithromycin, 14.1% for levofloxacin and 34.9% for metronidazole, with rates higher in Western/Central and Southern Europe than in Northern Europe. A steady increase in clarithromycin resistance and almost doubling of levofloxacin resistance was noted, linked with an increase in outpatient antibiotic usage[7,16,17]. As opposed to emerging resistance to previously mentioned antibiotics, resistance to amoxicillin, bismuth, furazolidone and tetracycline remains low[9]. The implication of antibiotic resistance is mainly accentuated with clarithromycin. In the case of clarithromycin resistance the rate of success of clarithromycin-containing regimen is very low (10%-30%). Metronidazole resistance is associated with 5%-25% lower eradication rate[10,18]. Levofloxacin, as metronidazole resistance can be overcome with increasing the length of treatment and using bismuth in quadruple therapy[16,19]. Other contributing factors are the presence of dormant coccoid bacterial forms, the density of *H. pylori* in the stomach, virulence factor status, biofilm formation, intracellular location of the bacteria and the presence of multistrain infection[13-15,20,21]. The presence of dormant, non-replicating bacteria causes phenotypic resistance, which is a form of reversible antibiotic resistance causing treatment failure[9].

FIRST LINE ESTABLISHED AND NOVEL TREATMENT OPTIONS

Standard triple therapy (proton pump inhibitor-PPI, clarithromycin, amoxicillin or metronidazole for 7-14 d) was a cornerstone of *H. pylori* treatment for years, recently gaining unacceptable eradication rates, mainly due to increase in clarithromycin resistance. According to new Maastricht guidelines, it is not advisable to use standard triple therapy in areas with clarithromycin resistance over 15%-20%, what lead to revival of some forgotten regimens, and construction and evolution of new ones[6,8]. Considering the fact that it is possible to overcome the resistance to metronidazole and that resistance to certain antibiotics rarely develops, new eradication schemes of various duration and dosage with strong inhibition of acid secretion, have been constructed to bypass triple therapy failure[16].

Bismuth-containing quadruple therapy represents alternative to standard triple therapy in areas with low clarithromycin resistance and the main first-line therapeutic option for areas with high prevalence of clarithromycin resistance. Consisting of bismuth salt, tetracycline and metronidazole it is effective independently of clarithromycin resistance. By using this regimen at full doses and for 14 d one can expect 95% or greater treatment success, irrespective of the level of metronidazole resistance[22]. Therapy for 7, and likely 10 d is very susceptible to metronidazole resistance, however, the prevalence of resistance, which results in a decrease in outcome to less than 90%, is probably approximately 30%[18]. Unfortunately recent meta-analysis, evaluating empirical approach with bismuth-containing quadruple or standard triple eradication regimen, revealed suboptimal data (78.3% and 77% eradication therapy success)[23]. Possible negative effects of metronidazole resistance could be resolved with increased dosage and longer duration of treatment (10-14 d). This regimen has been sometimes marginalized through literature considering the unavail-

ability of bismuth salts and tetracycline worldwide, complex dosing scheme, not yet known optimal dose and adverse effects[6]. After a study by Malfertheiner *et al*[24] and Venerito *et al*[25] in 2011, a revival of a quadruple regime consisting of PPI with a 3 in 1 capsule containing bismuth subcitrate potassium, metronidazole and tetracycline has emerged with improved eradication success exceeding 90%.

The PPI component of *H. pylori* eradication regimens has a major influence on overall therapy success, due to its intrinsic antibacterial effect, and more importantly the possibility to convert the nonproliferating, dormant forms of bacteria into active, proliferating forms by raising pH, leading to higher antibiotic efficacy[26]. Recent studies with new generation PPIs (esomeprazole and rabeprazole) have shown their higher potency in raising pH irrespective of CYP2C19 polymorphism, with eradication rate improvement[27,28].

First developed in Italy in the 90s, sequential therapy (5 d PPI and amoxicillin, followed by 5 d PPI, clarithromycin and metronidazole), is a regime that was proven to be more efficacious than triple therapy in many studies[8,29-32]. Recent multicenter randomized trial in Taiwan showed superiority of sequential over standard triple therapy, adding to its confirmation on population outside Italy[33]. The ability to eradicate the clarithromycin resistant bacteria has been demonstrated, and a sequential therapy has been already included in the recent consensus report as a valid first line option in geographic regions with high clarithromycin resistance[6]. Possible limitation of this regimen could be related to inferior results in treatment of dual resistant strains[30]. The level of metronidazole resistance determines the level of clarithromycin resistance required for eradication regimen success to decrease to < 90%. In low clarithromycin resistance areas metronidazole resistance undermines 10 d sequential therapy when it reaches 20%, and 14 d sequential therapy at approximately 30%. If metronidazole resistance is absent or low, sequential therapy for 10 or 14 d is very mildly affected with clarithromycin resistance. The complexity of sequential therapy was frequently reported as a possible problem of that regimen. Since this issue was never objectively evaluated, comparative studies of different therapy regimens (by using questionnaire, by prescriptive time evaluation *etc.*) are needed. The mechanism behind sequential therapy success is thought to be the disruption of the bacterial wall with initial 5 d of amoxicillin therapy, what prevents formation of efflux channels and consequent resistance for clarithromycin[15,34]. Nevertheless, a lot of authors have been hypothesizing that the use of 3 antibiotics, rather than the sequential scheme of administration, adds to this regime efficacy[15,35,36]. In this situation, adding to complexity and possible lower adherence to therapy, complexity of sequential administration would be unnecessary.

Two novel eradication regimens, namely concomitant (PPI, clarithromycin, amoxicillin, metronidazole for 10 d) and hybrid (PPI and amoxicillin for 7 d, followed by PPI, amoxicillin, clarithromycin and metronidazole for 7 d), have proven their efficacy over triple and sequential therapy in the last few years, especially with dual (clarithromycin and metronidazole) resistant strains[15,37-43]. Several studies have demonstrated that concomitant therapy is more effective (with comparable side effects) than standard triple therapy, although highly variable efficacy is reported with concomitant therapy and optimal treatment duration is not defined[36,44]. Most of these studies were performed with shorter (5-7 d) schemes. Meta-analyses and recent head-to-head comparison in Thailand have shown that the outcome of concomitant therapy is duration dependent[44-46]. Unsatisfactory results have been recently reported even when this scheme was adopted for two weeks. One of the findings of a large randomized trial in Latin America showed that 14 d triple therapy is more effective than 5 d concomitant or 10 d sequential therapy[47]. Geographic variations in the pattern of *H. pylori* resistance to antibiotics might account for some of these discrepancies in results, accentuating the need for implementation of local resistance pattern knowledge in generalized eradication therapy recommendations. Some of the advantages of non-bismuth quadruple regimens over the sequential regimen are longer duration of all the prescribed antibiotics, and broader validation in randomized controlled trials (RCT) of wider geographical regions[39]. A recent RCT in Spain over 338 patients, showed a slight advantage of concomitant over sequential therapy, allowing close to 90% eradication rates[34]. Non-bismuth eradication schemes are recommended as first line therapy in areas with high clarithromycin resistance (> 15%-20%) where bismuth containing quadruple therapy is not locally available[6]. The problem with empirical application of concomitant regimen is dual metronidazole-clarithromycin resistance. Before final conclusions on topic of concurrent *vs* sequential regimen further wider geographical region studies are essential in populations with high dual resistance.

A recently proposed hybrid therapy has been proven equally effective to 14 d concomitant regimen in a pilot study (ITT eradication rate 90% *vs* 92%)[37]. Therefore it could be considered in the same populations where concomitant therapy is recommended. This is a novel regimen, with only few evaluation studies published, so the optimal therapy duration and success rates in populations with high dual resistance are still not defined. This protocol is more complicated than those earlier proposed, with retained complex sequential approach and possible higher side effects rates because of concomitant treatment in the second phase. According to the preliminary data it is associated with comparable side effect profile and therefore merits consideration in further comparative studies in order to define final conclusions[10].

When considering an eradication protocol in first, and especially multiple therapy line failure, it is of utmost importance to reassess patients compliance and prior antibiotic usage, to design a scheme according to local resistance patterns and therapy success, and always confirm successful eradication[6,7,9,10,15]. Because of emergence

of resistance to previously used antibiotic, it is advisable not to use the same antibiotic after therapy failure, and to modify the scheme duration, dosage and antisecretory effect, with addition of antibiotics for which resistance rarely develops[48,49]. After second line treatment failure it is recommended to perform culture and susceptibility testing prior to third line treatment[6]. An expert agreement is that culture susceptibility testing before first or second line therapy is not advisable. With known obstacles of culture susceptibility testing, such as the need for endoscopic examination, the fact that culture is time-consuming, costly and not 100% sensitive and that susceptibility knowledge is not always followed with accordant eradication, highly effective empiric first line and rescue regimens can achieve acceptable results[8,15,50].

RESCUE REGIMENS

Several rescue regimens have been evaluated in recent years, especially with worldwide rise of clarithromycin and metronidazole resistance. Bismuth quadruple regimes are recommended as first and second line treatment options in areas of high clarithromycin resistance[6]. Based on analysis of 30 studies, after first line standard triple regimen, mean second line bismuth quadruple regimes yielded 77% eradication therapy success. Major determinants of eradication efficacy were metronidazole dosage and treatment duration[49].

Another rescue regimen recommended by the Maastricht guidelines for second and third line therapy, is the triple levofloxacin protocol (PPI, levofloxacin and amoxicillin for 10-14 d)[6]. It is especially valuable in situations where sequential and concomitant regimens where used, and where highly possibly dual resistant bacteria emerged[49,51]. Recently Gisbert *et al*[52] evaluated levofloxacin rescue therapy after sequential and concomitant treatment, and presented an encouraging eradication rate of 75%. When comparing levofloxacin triple therapy to bismuth quadruple therapy, which fails in around 20%-30% of patients, several meta-analyses have shown better outcomes with the levofloxacin regimen[48,53]. Besides being established as a rescue protocol, levofloxacin based therapy has proven its efficacy as a first line treatment, although not generally recommended due to rapid emergence of fluoroquinolone resistance and adverse effects[8,15,53]. In contrast to metronidazole there is no dose dependent-effect in overcoming levofloxacin resistance (eradication protocols becomes ineffective when resistance reaches 13%). In a randomized controlled study by Federico *et al*[35], a 5 d levofloxacin concomitant and 10 d sequential scheme in treatment naïve patients, achieved per-protocol eradication rates of 96.5% and 95.5%, respectively. An interesting study from Australia, evaluating fluoroquinolone containing regimens, showed high eradication rates around 95% with quadruple therapy consisting of PPI, amoxicillin or bismuth subcitrate, rifabutin and ciprofloxacin, in patients who failed at least one therapy course[54]. Considering overall resistance issues, possibly better fluoroquinolone-containing regimens include fluoroquinolone-bismuth therapy and fluoroquinolone concomitant therapy. Since neither of this protocol has been optimized or tested widely, generally they should be used as tailored therapies especially in the context of the emerging levofloxacin resistance[10].

Aside from tailored therapy according to culture susceptibility testing, few antibiotics with practically inexistent resistance have been evaluated. Rifabutin, a rifamycin derivate commonly used to treat *Mycobacterium avium-intracellulare*, has shown promising eradication rates in patients with several therapy failures. The eradication rates for second-, third- and forth and more line therapies are 79%, 66% and 70% respectively[55]. It is marginalized in *H. pylori* treatment due to its high cost, severe side effects, primarily myelotoxicity, and possibility of multiresistant *Mycobacterium tuberculosis* emergence[56]. Additional studies are needed to optimize the regimen in terms of dose and duration. Due to low primary bacterial resistance, furazolidone was evaluated as a multiple failure rescue regimen, but has not found an established position, mainly due to its possible genotoxic and carcinogenic effect[57].

WHAT THE FUTURE BRINGS?

As evident from myriad emerging studies evaluating established and creating new *H. pylori* eradication schemes recently, a consensus therapy has not yet been achieved. Main factors for eradication therapy failure are antibiotic resistance and poor patient compliance[10]. With regard to before mentioned culture susceptibility testing deficiencies and highly effective first and second line empirical treatments, the so-called "tailored" eradication therapy has not yet taken effect. With the development of non-invasive, rapid and culture-free susceptibility tests, primarily for clarithromycin and levofloxacin, it would be easier to perform tailored therapy even for treatment of naïve patients, with achievement of originally expected eradication rate of > 95%[9,17]. The use of polymerase chain reaction, a fast and inexpensive, culture-free method for susceptibility testing, was proven to improve the eradication rate when used to create tailored therapy[58]. Recently a study from Taiwan was published, evaluating genotypic resistance-guided third-line sequential therapy and showing promising eradication results. Genotypic resistance testing is more convenient and rapid than standard culture susceptibility testing, with a possibility to determine resistance even from stool samples[59]. With more efficient susceptibility testing, local resistance surveillance would be easier to perform, adding to higher success of eradication therapy in general[7,9]. While waiting for generally available and reliable noninvasive susceptibility tests, allowing unrestricted application of tailored therapy, we should keep in mind that success rate of proposed regimens justify in many instances empirical application of first- and second- line therapies. Even with possibility that sometimes local or regional expert treatment guidelines will not be strictly in line with gen-

eral ones, optimal approach is to use regimens that have been proven to be reliably excellent locally and/or regionally. Expert decisions should be based on knowledge of regional resistance patterns (obtained from imperative regional antimicrobial surveillance programs), local clinical experience with regard to which regimens are best effective and available and history of prior patient drug exposure. The regimen of the highest predicted success rate should apply with confirmation of successful eradication outcome. This approach allows us screening for possible increase in appearance of antimicrobial resistance in everyday clinical practice.

Besides the importance of knowing detailed patient antibiotic usage history and the ability of the physician to persuade the patient to take the medicines according to plan, a lot of effort is being made to reduce eradication therapy side effects. Various probiotic compounds containing *Lactobacillus*, *Bifidobacterium*, *Saccharomyces* and other benevolent bacteria, have been evaluated in improving eradication rate and reducing therapy side effects. Their contribution to eradication therapy is accomplished through direct antibacterial effect, modulation of host immune response and stabilization of the mucosal barrier[60,61]. Few recent studies have shown beneficial effect of probiotic compounds on eradication rate and on diminishing therapy side effects, although results from different studies are contradictory, suggesting a need for further evaluation[61-63].

Development of a vaccine against *H. pylori* infection is an ultimate goal for eradicating all the negative effects of this versatile bacterium. There are several reasons for the fact that an efficient vaccine has not yet been developed. It took many years for the scientific community to acknowledge the contribution of *H. pylori* in peptic ulcer disease, and especially its carcinogenic potential. Another important factor is the complicated host immune response, along with considerable genetic diversity of *H. pylori*, what hampers the technical path in developing the vaccine. At last, due to a long period between *H. pylori* acquisition and manifestation of disease, especially gastric cancer, there is a general climate making development of the vaccine slow and inefficient[64,65].

CONCLUSION

A simple and uncomplicated path in *H. pylori* eradication has been recently disturbed with progressive bacterial resistance for cornerstone antibiotics. With the development and implementation of novel eradication regimens the situation looks more promising, although the final answer to *H. pylori* infection has not yet been established. Hopefully, non-invasive and rapid susceptibility tests will take place in strengthening the tailored therapy concept, and efficient vaccine will obviate the need for *H. pylori* induced disease management.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (6): *Helicobacter pylori*

Helicobacter pylori-negative, non-steroidal anti-inflammatory drug: Negative idiopathic ulcers in Asia

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Abstract

Since the discovery of *Helicobacter pylori* (*H. pylori*) infection in the stomach, the bacteria infection and non-steroidal anti-inflammatory drugs (NSAIDs) use had been considered to be the 2 main causes of peptic ulcers. However, there have been recent reports of an increase in the proportion of peptic ulcers without these known risk factors; these are termed idiopathic peptic ulcers. Such trend was firstly indicated in 1990s from some reports in North America. In Asia, numerous studies reported that idiopathic ulcers accounted for a small percentage of all ulcers in the 1990s, but in the 2000s, multiple studies reported that the proportion of idiopathic ulcers had reached 10%-30%, indicating that the incidence of idiopathic ulcers in Asia has also been rising in recent years. While a decline in *H. pylori* infection rates of general population in Asia is seen as the main reason for the increased incidence of idiopathic ulcers, it is also possible that the absolute number of idiopathic ulcer cases has increased. Advanced age, serious systemic complication, and psychological stress are considered to be the potential risk factors for idiopathic ulcers. Management of idiopathic ulcers is challenging, at present, because there is no effective preventative measure against recurrence in contrast with cases of *H. pylori*-positive ulcers and NSAIDs-induced ulcers. As it is expected that *H. pylori* infection rates in Asia will decline further in the future, measures to treat idiopathic ulcers will also likely become more important.

Key words: *Helicobacter pylori*; Non-steroidal anti-inflammatory drugs; Idiopathic peptic ulcer

Core tip: In Asia, numerous studies reported that idiopathic ulcers accounted for a small percentage of all ulcers in the 1990s, but in the 2000s, multiple studies reported that the proportion of idiopathic ulcers had reached 10%-30%, indicating that the incidence of idiopathic ulcers in Asia has also been rising in recent years. As it is expected that *Helicobacter pylori* infection rates in Asia will decline further in the future, measures to treat idiopathic ulcers will also likely become more important.

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INTRODUCTION

After *Helicobacter pylori* (*H. pylori*) was discovered in 1982, it was considered to be the cause of a large number of peptic ulcers (90%-95% of duodenal and 70%-90% of

gastric ulcers)[1]. *H. pylori*, aspirin, and other non-steroidal anti-inflammatory drugs (NSAIDs) comprise the causes of a large proportion of peptic ulcers. A subsequent rise in global NSAID use and a relative increase in the proportion of NSAID-caused ulcers[2,3] led to wide acceptance that *H. pylori* infection and NSAID use are the 2 main causes of peptic ulcers[4]. Other causes include Zollinger-Ellison syndrome, Crohn's disease, and viral infections such as cytomegalovirus and herpes.

However, there have been recent reports of an increase in the proportion of peptic ulcers without these known risk factors; these are termed idiopathic peptic ulcers. By the 1990s, several studies reported that idiopathic ulcers comprised 20%-40% of all peptic ulcers in North America[5-7]. In this region, 20% of *H. pylori*-positive ulcers recurred after bacterial elimination[8], a markedly higher percentage than that reported in other areas. This finding indicated that a large number of "bystander" cases existed, wherein a patient is positive for *H. pylori* but the bacteria are not directly involved in causing the ulcer. In the 2000s, increases in the proportion of idiopathic ulcers were also reported in Europe and Asia (Table 1). However, a few studies still report low idiopathic ulcer proportions (4%), including a recent report from Italy[6].

Detailed reviews of idiopathic ulcers were published in 2002[37] and 2008[38]. Here, we examine more recent trends in the incidence of idiopathic ulcers, particularly in Asia.

PRECAUTIONS FOR IDIOPATHIC ULCER DIAGNOSIS

When diagnosing idiopathic ulcers, *H. pylori* infection and history of NSAID use, the 2 main causes of peptic ulcers, must be completely ruled out. Otherwise, one will naturally find a high proportion of idiopathic ulcers.

Various methods are used to diagnose *H. pylori* infection, none of which is 100% accurate alone. Thus, the absence of *H. pylori* can be determined only when several tests are combined and the results of all are found to be negative[38]. Diagnostic methods that use endoscopic biopsy specimens-histology, culture, and rapid urease tests (RUT)-can show false negatives due to sampling errors caused by non-uniform distribution of *H. pylori* inside the stomach[39]. Therefore, biopsies must be obtained from several locations. Further, these methods can show false negatives immediately after acute upper digestive tract hemorrhage[40]. In these situations, other diagnostic methods must be used in combination. The urea breath test (UBT) examines urease activity throughout the stomach due to *H. pylori*, and is used to compensate for sampling errors in tests using biopsy specimens. However, care is needed when using UBT, as false negatives can result when bacterial concentrations decrease during administration of proton pump inhibitors (PPIs)[41], similar to other tests using biopsies. The serum antibody method is not affected by PPI administration or acute upper digestive tract hemorrhage; therefore, it is useful for diagnosing *H. pylori* infection in these situations. However, this method continues to show positive results for some time after bacterial elimination[42], making it difficult to differentiate between current and past infections. Nevertheless, it is a useful test because it provides a precise diagnosis of idiopathic ulcer (as few false negatives as possible). There are several tests for even more precise diagnosis of *H. pylori*-negative patients; although these tests do not directly confirm the presence of *H. pylori*, they eliminate the diagnosis of idiopathic ulcer based on tissue findings associated with infection, such as neutrophil infiltration into the gastric mucosa or atrophy of the gastric mucosa[28,30].

Another important factor in diagnosing idiopathic ulcers is careful elimination of NSAID users. Aspirin and other NSAIDs can be purchased without a doctor's prescription in many countries. A large number of surreptitious NSAID users likely exist; therefore, patients must be questioned scrupulously about their drug history. Indeed, several previous studies have clarified the existence of surreptitious NSAIDs users. Lanas *et al*[43] examined patients with gastrointestinal perforation using platelet cyclooxygenase activity in the blood as an objective marker of aspirin usage. They identified 13% more aspirin users with this method than were found through investigation of medical history. Moreover, based on measurements of blood salicylic acid concentrations, Hirschowitz *et al*[44] reported that 50% of intractable peptic ulcer patients who denied using aspirin were in fact aspirin users. These findings underscore the necessity of carefully eliminating NSAID users when diagnosing idiopathic ulcers. Retrospective studies, which can only determine NSAID usage from past medical records, would be inevitably too lenient in eliminating NSAID users. The result would be a tendency to report higher rates of idiopathic ulcers; therefore, care must be taken when interpreting such data.

IDIOPATHIC ULCER TRENDS IN ASIA

Six reports of the proportion of idiopathic ulcers in Asia were published in 1999-2003 based on patient data from the 1990s[19-24], 5 of which reported low rates of 1.3%-4.1%[19-23]. In 9 studies from 2005-2006 based on patient data from the 2000s[28-36], almost all reported that the proportion of idiopathic ulcers was 10%-30%, indicating that the proportion of these ulcers among all peptic ulcers in Asia is increasing. A decline in *H. pylori* infection rates among background healthy individuals is likely a cause of this increase. This trend is common among Asian countries[45], and indicates that recent improvements in sanitation and the increased use of *H. pylori* elimination therapies have decreased the *H. pylori* infection rates in the region. Even if the increase in idiopathic ulcers is merely a relative rise accompanying a decline in *H. pylori*-positive ulcers, or if *H. pylori* has only been coexisting as a bystander in many cases, the decline in *H. pylori* infection rates among the overall population has given prominence to the issue of idiopathic ulcers. Further, it

Table 1 Prevalence of idiopathic ulcers

Ref.	Year	Country	Sampling period	Sampling style	Subjects disease	Subjects number	Prevalence of IPU (%)	Diagnostic method for *H. pylori*
North America								
Perterson *et al*[5]	1996	United States	Not mentioned	RCT	DU	185	26	Hist/RUT/culture
Jyotheeswaran *et al*[6]	1998	United States	1993-1996	Retrospective	PUD	305	39	Hist/RUT
Ciociola *et al*[7]	1999	United States	1991-1995	6 RCT	DU	2910	27	Hist/RUT/culture
Europe								
McColl *et al*[9]	1993	United Kingdom	Past 5 yr	Cross-sectional	DU	400	1.5	Hist/RUT/UBT/antibody
Gisbert *et al*[10]	1999	Spain	Not mentioned	Prospective	DU	774	0.8	RUT/culture/UBT
Meucci *et al*[11]	2000	Italy	1995-1996	Prospective	PUD	409	4.4	Hist/RUT
Bytzer *et al*[12]	2001	Denmark	1993-1995	RCT	DU	276	8	Hist/culture/antibody
Konturek *et al*[13]	2003	Poland	1996-2000	Prospective	PUD	1898	18.7	UBT
Arents *et al*[14]	2004	Netherland	1991-1998	Retrospective	PUD	405	5	Hist/RUT/culture
Arroyo *et al*[15]	2004	Spain	Not mentioned	Prospective	PUD	830	4.1	Hist/RUT/UBT
Sbrozzi-Vanni *et al*[16]	2010	Italy	2005-2007	Retrospective	PUD	300	4	Hist/UBT
Musumba *et al*[3]	2012	United Kingdom	2005-2010	Retrospective and prospective	PUD	386	12	Hist/RUT/antibody
Australia								
Borody *et al*[17]	1991	Australia	Not mentioned	Prospective	DU	302	0.3	Hist/culture
Xia[18]	2000	Australia	Not mentioned	Prospective	PUD	48	40	Hist/RUT/culture
Asia								
Tsuji *et al*[19]	1999	Japan	1995-1997	Prospective	DU and GU	DU: 120, GU: 215	GU:2.3, DU: 0.9	Hist/RUT/culture/antibody
Higuchi *et al*[20]	1999	Japan	Not mentioned	Cross-sectional	DU	338	2.4	Hist/RUT/culture/UBT/antibody
Aoyama *et al*[21]	2000	Japan	1995-1998	Cross-sectional	DU and GU	302	2.6	Hist/culture/antibody
Nishikawa *et al*[22]	2000	Japan	1992-1997	Cross-sectional	PUD	398	1.3	Hist/RUT/antibody
Chan *et al*[23]	2001	Hong Kong	1997-1998	Prospective	hPUD	977	4.1	Hist/RUT
Xia *et al*[24]	2001	Hong Kong	1997-1999	Prospective	DU	599	17.4	Hist/RUT/UBT
Kamada *et al*[25]	2003	Japan	Past 8 yr	Cross-sectional	DU	464	1.3	Hist//UBT/antibody
Chu *et al*[26]	2005	Hong Kong	1996-2002	Prospective	DU	1343	23	Hist/RUT
Yakoob *et al*[27]	2005	Pakistan	1999-2000	Retrospective	DU	217	29	Hist/RUT
Hung *et al*[28]	2005	Hong Kong	2000	Prospective	hPUD	638	18.8	Hist/RUT
Ong *et al*[29]	2006	Singapore	2002-2004	Prospective	PUD	600	8	Hist/RUT
Ootani *et al*[30]	2006	Japan	2000-2002	Prospective	hPUD	116	1.7	RUT/UBT/antibody
Jang *et al*[31]	2008	South Korea	2004-2005	Prospective	PUD	895	22.2	Hist/RUT
Chen *et al*[32]	2010	Taiwan	2003-2004	Prospective	DU	731	8	RUT/UBT
Goenka *et al*[33]	2011	India	2008-2009	Prospective	PUD	142	36.6	RUT/UBT
Chang *et al*[34]	2011	Taiwan	2007-2008	Prospective	PUD	204	17.2	Hist/RUT/UBT
Wong *et al*[35]	2012	Hong Kong	2002-2009	Prospective	hPUD	4827	13.8	Hist/RUT
Kang *et al*[36]	2012	South Korea	2006-2008	Prospective	PUD	173	16.2	Hist/RUT/culture/antibody

RCT: Randomized controlled trial; RUT: Rapid urease test; UBT: Urea breath test; PUD: Peptic ulcer diseases; DU: Duodenal ulcers; GU: Gastric ulcers; hPUD: Hemorrhagic peptic ulcer diseases; *H. pylori*: *Helicobacter pylori*.

has been reported that not only the proportion of idiopathic ulcers has increased but also the actual number of cases has been increasing annually[28]. This trend suggests the existence of not just a relative cause but also some other direct factor that is contributing to the incidence of idiopathic ulcers. Next, we will examine the incidence of idiopathic ulcers in various Asian countries in detail.

Hong Kong

Hong Kong has been the most active region in conducting clinical studies on idiopathic ulcers; these studies have provided valuable data for understanding trends in idiopathic ulcer incidence. Two studies on the proportion of idiopathic ulcers based on patient data from the 1990s reported somewhat scattered results of 4% and 17%[23,24]. However, 3 studies based on data from the 2000s reported relatively high and consistent values of 14%-23%[26,28,34]. In particular, a recent major study on approximately 5000 peptic ulcer patients from 2002-2009 reported a 13.8% proportion of idiopathic ulcers[34], again showing that these ulcers are not rare in Hong Kong. A study on yearly changes in the proportion of idiopathic ulcers in the same institution reported a rise from 1997-1998 to 2000 of 4.1% to 18.8%[28], with other reports also showed an increase in the proportion of idiopathic ulcers over time[26]. While this rise in idiopathic ulcer rates reflects a relative increase accompanying a decline in *H. pylori*-positive ulcers due to the increased use of bacterial elimination therapy or decline of *H. pylori* infection rate among background healthy population[46], yearly data also suggest that the actual number of idiopathic ulcer patients is increasing[28].

Japan

Five studies of the frequency of idiopathic ulcers in Japan based on 1990s patient data reported very low rates

of 0.9%-2.6%[19-22,24]. In addition, a 2000-2002 investigation of hemorrhagic ulcer patients found that 11% were *H. pylori*-negative and NSAID-negative[30]. However, after eliminating cases with a possible history of *H. pylori* infection based on histological atrophy of the gastric mucosa, the final proportion of cases with idiopathic ulcers was 1.7%[30]. There have since been no further studies on the frequency of idiopathic ulcers in Japan. Recently, there has been a marked decline in *H. pylori* infection rates among the general Japanese population[47], and it is possible there has been an accompanying rise in the proportion of idiopathic ulcers, as has occurred in other Asian countries. Sugiyama *et al*[48] found a similarly low proportion of idiopathic ulcers in Japanese ulcer patients divided into 2 age groups, which showed major differences in *H. pylori* infection rates. Based on this finding, they surmised that the proportion of idiopathic ulcers does not vary according to *H. pylori* infection rates. Considering the extremely low proportion of idiopathic ulcers in Japan in the 1990s, information on recent trends would be of great interest.

South Korea

There are two reports from South Korea on the frequency of idiopathic ulcers based on patient data from the 2000s. One of them, based on 2004-2005 data, reported a high (22%) proportion of idiopathic ulcers[31]. However, the diagnosis of *H. pylori* infection in this study was based on only a single RUT and histological examination. Therefore, it is unclear whether the diagnosis of *H. pylori* negativity was sufficiently precise. The more recent study, based on 2006-2008 data, employed a strict definition of *H. pylori* negativity using a urease test, histological examination, culture, and the serum antibody method. This report confirmed a high proportion of idiopathic ulcers (16.2%)[36]. No reports from South Korea are based on patient data from the 1990s; therefore, comparisons with past data cannot be performed. However, it can be surmised that idiopathic ulcers are not rare in South Korea, similar to Europe and the United States. The recent decline in *H. pylori* infection rates among the general South Korean population[49,50] can be considered as the cause of the increase in the proportion of idiopathic ulcers unrelated to *H. pylori*.

Taiwan

A study from Taiwan based on data from 2003-2004 reported an idiopathic ulcer frequency of 8%[32]. Another report based on 2007-2008 data reported a 17% frequency[34], indicating that the proportion of idiopathic ulcers has been rising in recent years. The cause of this trend is also thought to be a decline in *H. pylori* infection rates in Taiwan[33].

Other Asian countries

A study of 1999-2000 patient data from Pakistan reported that 29% of ulcers were idiopathic[27]. Although this developing country was thought to have a high *H. pylori* infection rate at the time[51], the study reported a large proportion of idiopathic ulcers. However, problems with the methods used to diagnose *H. pylori* infection and issues with the retrospective study design, which did not allow a precise idiopathic ulcer diagnosis, possibly led to this apparently high proportion.

A study of 2002-2004 patient data from Singapore reported an 11% proportion of idiopathic ulcers[29]. However, data on serum thromboxane B2 concentrations suggested that 1/3 of these cases were surreptitious NSAID users, resulting in a true proportion of idiopathic ulcers of 8%[29]. Singapore is a multi-ethnic country and is known to have relatively lower rates of *H. pylori* infection than other Asian nations[52]. Nevertheless, this final proportion of idiopathic ulcers is relatively low. This study showed the importance of eliminating surreptitious NSAID users through blood tests, as well as through interviews.

Patient data from India from 2008-2009 showed an extremely high proportion of idiopathic ulcers at 37%[33], on par with rates in North America. This study diagnosed *H. pylori* using RUT and UBT, thus having a certain level of precision. Although the *H. pylori* infection rates in India have traditionally been high[53,54], recent studies have reported a decline in infection rates[55], and the high frequency of idiopathic ulcers may be a reflection of this. However, since this study involved only a single institution and a relatively small number of ulcer patients, further investigation is needed.

RISK FACTORS OF IDIOPATHIC ULCERS

Although a clear cause of idiopathic ulcers hasn't yet to be shown, several factors related to the condition have been proposed.

Age

Numerous studies in Europe, the United States, and Asia have shown that idiopathic ulcer patients are significantly older than those with simple *H. pylori* ulcers or *H. pylori*/NSAID ulcers[11,23,24,28,29,56], although older age may be a mere confounder of the following risk factors such as systemic complications or psychological stress. Aging has been shown to be accompanied by a decline in the defense functions of the gastric mucosa owing to a variety of mechanisms, particularly a lower prostaglandin concentration in the gastric mucosa as a person ages[57], which may be a potential cause for gastric ulcers but not for duodenal ulcers. Prostaglandin plays a central role in the gastric mucosa defense structure by increasing gastric mucus secretions, bicarbonate secretions, and blood flow[58]. Thus, the diminished prostaglandin concentration in elderly individuals renders the gastric mucosa more fragile, creating an environment in which ulcers are more likely to occur.

Systemic complications

Many studies, primarily from Asia, have reported that a wide variety[22,24,26,29] of serious[35] systemic complications

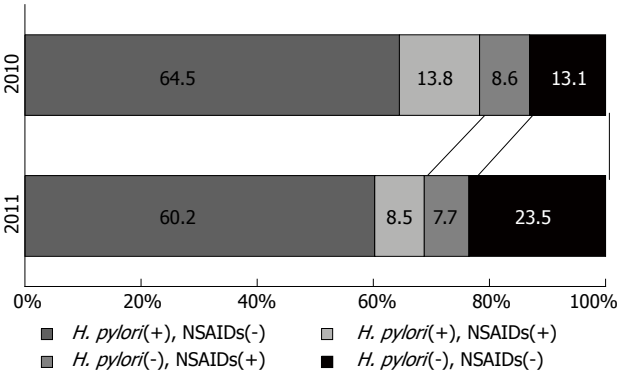

Figure 1 Changing pattern of the etiology of peptic ulcers before (2010) and after (2011) the Great East Japan earthquake. In the analysis of etiologic factors of the ulcers, cases from each year were classified into four groups according to the *Helicobacter pylori* (*H. pylori*) status and non-steroidal anti-inflammatory drugs (NSAIDs) intake. The proportion of *H. pylori*-negative non-NSAIDs takers among PU patients after the earthquake (2011) was significantly higher than in the previous year (13% in 2010 *vs* 24% in 2011, $P < 0.05$). The date available from reference[67].

are risk factors for idiopathic ulcers. This finding is possibly related to reports stating that peptic ulcers that occur in intensive care units or against a background of serious underlying disease are unrelated to *H. pylori* infection[59,60]. Physical or psychological (see below) stress caused by an underlying disease might be related to ulcer incidence.

Several recent studies have reported the relationship between hepatocirrhosis and hemorrhagic peptic ulcers[61-63]. In particular, decompensated hepatocirrhosis was demonstrated to be a risk factor for hemorrhagic peptic ulcer, independent of *H. pylori* infection[61-63]. Thus, hepatocirrhosis is an important cause of idiopathic ulcers. Although the pathology by which hepatocirrhosis leads to a peptic ulcer is complex, portal hypertension is likely involved. Visceral congestion from portal hypertension might be linked to ulcer incidence, as it damages the gastroduodenal mucosal blood flow and inhibits the process of mucosal repair[63-65]. In addition, decreased gastric prostaglandin synthesis observed in hepatocirrhosis patients may be involved in the hepatocirrhosis-related gastric mucosal injury[62,63].

Psychological stress

A link between psychological stress and peptic ulcers has long been suggested[65], but once *H. pylori* infection and damage from NSAID use were identified as the 2 main causes of peptic ulcers, it became unclear whether psychological stress should be considered an independent cause of peptic ulcer[4,21]. However, there have been reports of ulcer formation in the victims of the Great East Japan Earthquake that struck in 2011[67,68]. Kanno *et al*[67] compared the ulcer incidence 3 mo after the disaster with the ulcer incidence in the same period during the previous year; they found a 1.5-fold rise in ulcer cases after the disaster. Further, the proportion of *H. pylori*- and NSAID-negative (that is, idiopathic) ulcers rose significantly from 13% in 2010 to 24% after the disaster in 2011 (Figure 1)[67]. This study excluded cases complicated by severe trauma due to the disaster, showing indirectly that psychological stress can be an independent cause of peptic ulcers among disaster victims. Interestingly, the *H. pylori*-, NSAID-negative ulcers that arose after the disaster were in patients who were significantly older than those with other ulcer types[67]; this finding is consistent with the characteristic of idiopathic ulcers described above.

MANAGEMENT OF IDIOPATHIC ULCERS

Bacterial elimination therapy is often effective for preventing the recurrence of *H. pylori*-positive ulcers and does not require subsequent maintenance therapy with acid-suppressive agents[4]. In NSAID-induced ulcers, changing therapy to COX-2-selective NSAIDs or other alternative medications that do not cause as much damage to the gastrointestinal mucosa can be expected to suppress recurrences[4]. However, for idiopathic ulcers, although acid-suppressive agents can produce temporary relief, they are not an effective preventative measure against recurrence. It has been shown that recurrence rates are high when patients remain unmedicated after such temporary cures. A study from Denmark observed 32 unmedicated patients with *H. pylori*-negative duodenal ulcers for 2 years, and reported a 35% recurrence rate[12]. Additionally, a prospective study from Hong Kong that observed unmedicated patients with idiopathic, hemorrhagic gastroduodenal ulcers for 7 years found that 42% experienced a relapse of ulcer hemorrhage. This is 4 times the percentage found while observing patients with *H. pylori*-positive ulcers who were unmedicated after bacterial elimination[69]. Further, a recent report from South Korea found that compared to *H. pylori*-positive ulcers and NSAID-induced ulcers, idiopathic ulcers showed more recurrences, which led to increased medical costs[36].

Although it has been shown that recurrences can easily occur when patients with idiopathic ulcer are not treated, there is no consensus on whether maintenance therapy with acid-suppressive agents can effectively prevent these recurrences. In the aforementioned study from Denmark, PPI administration was found to have an effect on preventing the recurrence of *H. pylori*-negative duodenal ulcers[12]. However, a recent major follow-up study from Hong Kong that examined 663 patients with idiopathic hemorrhagic gastroduodenal ulcers did not find H_2-blocker or PPI administration to be effective in preventing ulcer hemorrhage relapse[35]. Around 50% of the cases in the Hong Kong study were gastric ulcers[35], and it was found that gastric ulcers, even idiopathic cases, might show different reactivity to acid-suppressive agents than duodenal ulcers. Until it is determined which medications can effectively prevent the recurrence of idiopathic ulcers, the most realistic choice appears to be continuous PPI therapy.

CONCLUSION

Numerous studies reported that idiopathic ulcers in Asia accounted for a small percentage of all ulcers in the 1990s, but in the 2000s, multiple studies reported that the proportion of idiopathic ulcers had reached 10%-30%. Despite limitations such as difficulties in precise diagnosis of *H. pylori* infection and identification of surreptitious NSAID users, it is clear that the incidence of idiopathic ulcers in Asia has been rising in recent years. While a decline in *H. pylori* infection rates in Asia is seen as the main reason for the increased incidence of idiopathic ulcers, it is also possible that the absolute number of idiopathic ulcer cases has increased. As it is expected that *H. pylori* infection rates in Asia will decline further in the future, measures to treat idiopathic ulcers will also likely become more important. Further multicenter studies from many different countries, with the same protocol, are necessary to investigate the real incidence of idiopathic ulcers and the real causes.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (6): *Helicobacter pylori*

Helicobacter pylori-associated immune thrombocytopenia: Clinical features and pathogenic mechanisms

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Abstract

Immune thrombocytopenia (ITP) is an autoimmune disease mediated by anti-platelet autoantibodies. There is growing evidence that the eradication of *Helicobacter pylori* (*H. pylori*) effectively increases platelet count in a considerable proportion of ITP patients infected with this bacterium. In the majority of ITP patients responding to *H. pylori* eradication therapy, the anti-platelet autoantibody response is completely resolved with no relapse for more than 7 years, indicating that the disease is cured. Therefore, adult patients with suspected ITP should be examined for *H. pylori* infection, and eradication therapy is recommended if the infection is present. Notably, however, the efficacy of *H. pylori* eradication therapy in ITP patients varies widely among countries, with a higher response rate in Japan compared with the United States and European countries other than Italy. The pathogenesis of *H. pylori*-associated ITP is still uncertain, although the mechanisms are known to involve multiple factors. *H. pylori* may modulate the Fcγ-receptor balance of monocytes/macrophages in favor of activating Fcγ receptors, and *H. pylori* components may mimic the molecular makeup of platelet antigens. Further studies of the pathogenic process of *H. pylori*-associated ITP may be useful for the development of new therapeutic strategies for ITP.

Key words: Autoantibody; Childhood; *Helicobacter pylori*; Fcγ receptor; Immune thrombocytopenia; Idiopathic thrombocytopenic purpura; Systemic lupus erythematosus

Core tip: In this review, we summarize recent updates on basic and clinical aspects of *Helicobacter pylori* (*H. pylori*)-associated immune thrombocytopenia (ITP). We highlight the efficacy of *H. pylori* eradication in adult and childhood ITP as well as in secondary ITP, variability in the efficacy of eradication in various countries, factors predicting the eradication-related platelet response, and the mechanisms responsible for the development of ITP in association with *H. pylori* infection. It is apparent that in a distinct subgroup of *H. pylori*-associated ITP, this bacterial infection is central to the ITP pathogenesis.

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INTRODUCTION

Helicobacter pylori (*H. pylori*), a gram-negative spiral bacterium, is the causative agent in chronic gastritis, gastric and duodenal ulcer disease, and gastric cancer. *H. pylori*

has also been implicated in the pathogenesis of extra-digestive disorders, including cardiovascular, hematologic, and autoimmune diseases[1]. The strongest evidence has been reported for immune thrombocytopenia (ITP), with high-quality studies showing that the disease improved after *H. pylori* was successfully eradicated.

ITP is a typical organ-specific autoimmune disease; it is mediated by anti-platelet autoantibodies that bind to platelets and megakaryocytes, accelerating platelet destruction by the reticuloendothelial system and suppressing platelet production[2]. The autoantibody response primarily targets platelet-surface glycoproteins such as GP Ⅱb/Ⅲa and GP Ⅰb. This condition is known as primary ITP when it occurs without an underlying disease, but it is also seen in patients with various diseases, including systemic lupus erythematosus (SLE). Although the etiology of ITP is obscure, microorganisms such as human immunodeficiency virus and hepatitis C virus are known to contribute to its development[3], indicating that in a particular subset of ITP, infectious agents play a significant role in the pathogenesis of the autoimmune response.

First observed in 1988[4], the increase in platelet counts in ITP patients after eradicating *H. pylori* has since been confirmed by several studies. Consequently, *H. pylori* eradication therapy is now a treatment option for ITP[5]. However, a number of questions regarding the relationship between *H. pylori* infection and ITP remained unsolved, including the great variability in the efficacy of *H. pylori* eradication therapy among countries, factors predicting the platelet response after *H. pylori* eradication, and mechanisms responsible for the platelet response associated with *H. pylori* eradication[6]. Some of these questions have been answered in the past few years. This review summarizes recent updates on clinical and therapeutic aspects of *H. pylori*-associated ITP, as well as the pathogenesis of this disease.

EFFICACY OF *H. PYLORI* ERADICATION IN ITP

Adult ITP

In 1988, Gasbarrini *et al*[4] reported that platelet counts increased in all of 8 *H. pylori*-infected patients with ITP who were treated with a regimen to eradicate *H. pylori*, while the platelet counts were unchanged in 3 *H. pylori*-infected patients who did not receive the regimen. Reports followed of partial or complete platelet responses observed in a large proportion of ITP patients treated with an *H. pylori* eradication regimen consisting of a 1- to 2-wk standard triple therapy with a proton pump inhibitor, clarithromycin, and either amoxicillin or metronidazole[6-8]. A nation-wide survey in Japan involved 207 *H. pylori*-infected adult patients with ITP, making it the largest study on the efficacy of eradicating *H. pylori* in ITP patients[9]. In that study, after the successful eradication of *H. pylori*, 63% of the patients achieved some degree of platelet recovery, and within this group, 23% showed complete remission at 12 mo after the eradication. Although most early studies excluded patients with severe thrombocytopenia, who were at high risk of bleeding, several case series have reported the efficacy of eradicating *H. pylori* even in patients with refractory ITP, including patients with severe thrombocytopenia that resisted multiple therapeutic regimens including splenectomy[9-11]. Although most studies began assessing the platelet counts one month after starting eradication therapy, we observed platelet recovery after just a week in almost half of those responding[12]. Long-term follow-up studies showed that this platelet response lasted 7 or more years after *H. pylori* was eradicated, with very few cases of relapse[13,14]. An assessment of circulating B cells producing anti-GP Ⅱb/Ⅲa antibodies before and after eradication treatment indicated that the anti-platelet autoantibodies disappeared after platelet recovery when *H. pylori* had been successfully eradicated[12]. Thus, in certain patients, ITP appears to be clinically and immunologically cured by eradicating *H. pylori*.

In the first systematic review with meta-analysis, Franchini *et al*[15] reviewed 788 patients with ITP, including 494 *H pylori*-infected patients collected from 17 studies, in 2007. Platelet counts increased in ITP patients who received eradication treatment, compared with untreated patients, and the weighted mean difference (WMD) in platelet count was 34.0×10^9/L regardless of the outcome of *H. pylori* eradication. The platelet counts increased significantly in *H. pylori*-infected patients after successful *H. pylori* eradication, compared with the following groups: untreated *H. pylori*-infected patients (WMD of 40.8×10^9/L), *H. pylori*-infected patients who failed eradication (WMD of 52.2×10^9/L), and *H. pylori*-uninfected patients (WMD of 46.4×10^9/L). Another systematic literature review involving 1555 patients revealed a weighted mean complete response (platelet count $\geqslant 100 \times 10^9$/L) after successful *H. pylori* eradication of 42.7%, and an overall response (platelet count $\geqslant 30 \times 10^9$/L, and at least doubling of the basal count) of 50.3%[16]. Even in patients with a low baseline platelet count ($< 30 \times 10^9$/L), the overall response rate was 35.2%, including 20.1% with a complete response. These findings indicate that *H. pylori* eradication is closely related to platelet recovery in adult ITP patients. Finally, another systematic review evaluated the efficacy of the *H. pylori* eradication regimen in patients with ITP by comparing the platelet response in patients with or without an *H. pylori* infection[17]. The odds of achieving platelet recovery following the eradication regimen were 14.5 times higher in 205 *H. pylori*-infected patients than in 77 *H. pylori*-uninfected patients. This clearly indicates that platelet recovery after the eradication regimen results from the eradication of *H. pylori* itself, rather than from *H. pylori*-independent mechanisms such as immune-modulatory effects of the drugs themselves, or the eradication of bacteria other than *H. pylori*.

The clear linkage between platelet recovery and the disappearance of *H. pylori* suggests a direct role for *H. py-*

lori infection in the ITP pathogenesis. In addition, in the majority of ITP patients who achieved a complete platelet response after *H. pylori* eradication, the anti-platelet autoantibody response was also eliminated. Thus, patients who are infected with *H. pylori* and who respond to the eradication therapy fall into a distinct, widely recognized ITP subgroup, termed *H. pylori*-associated ITP[18,19], that is considered a type of secondary ITP[5]. Given the relatively high efficacy, safety, and economy of *H. pylori* eradication therapy, *H. pylori* detection should be considered when examining adult patients who are suspected to have ITP, and eradication therapy is recommended if *H. pylori* infection is present[5,8].

Variability among countries

Although the efficacy of *H. pylori* eradication therapy in infected adults with ITP has been confirmed by high-quality systematic reviews, some studies have reported little to no platelet response after *H. pylori* eradication therapy. For example, Jarque *et al*[20] and Ahn *et al*[21] observed platelet recovery was in only 13% and 7%, respectively, of adult patients with ITP after successfully eradicating *H. pylori*. Michel *et al*[22] found no platelet response in 14 *H. pylori*-infected ITP patients even after successfully eradicating the *H. pylori*. These widely varying reports of the efficacy of *H. pylori* eradication therapy in ITP patients are explained, in part, by different eligibility criteria and different definitions of platelet response among the various studies. However, response rates differed between the 57.9% reported by studies in Japan, and the 38.3% rate reported by studies from other countries[16]. Studies from Japan and Italy tend toward better response rates, ranging from 28% to 100%, than studies from the United States and Spain (< 13).

Thus, variability in the efficacy of *H. pylori* eradication in different populations should be considered to establish recommendations for screening and eradicating *H. pylori*. For example, *H. pylori* screening is certainly worthwhile in Japan, a country with a high prevalence of infection and a high response rate to eradication treatment. In fact, in a recently developed reference guide for managing adult ITP in Japan, *H. pylori* eradication is a prominent strategy for managing ITP in adult patients[23]. Namely, the guide states that all patients diagnosed with ITP should be screened for *H. pylori* infection, and eradication therapy is recommended as a first line of treatment, regardless of platelet count, if *H. pylori* infection is present. These recommendations may not be appropriate in the United States or in European countries (other than Italy), in which both the prevalence of infection and the response rates to eradication therapy are low. Indeed, *H. pylori* eradication is not mentioned in the international consensus report on the management of primary ITP[24], while the eradication therapy is recommended in patients who are found to have *H. pylori* infection in the American Society of Hematology 2011 evidence-based practice guideline for ITP[5].

A recent systematic review demonstrated a correlation in ITP patients between the prevalence of *H. pylori* infection and the platelet response rates to eradication therapy[16]. The reason for such variability among countries is not clear, but differences in the epidemic *H. pylori* strains according to geographical area could account for differences in the clinical response. In this regard, *H. pylori* strains that possess cytotoxin-associated gene A (CagA) have been proposed to have a role in the pathogenesis of *H. pylori*-associated ITP based on the potential cross-reactivity between CagA and platelet glycoproteins[25,26]. The frequency of CagA-positive strains varies by geographic location: the majority of the *H. pylori* strains found in Eastern Asia, including Japan, express CagA, whereas the proportion of CagA-positive strains in European countries and North America is much lower[27]. However, genetic and other environmental factors are also likely to contribute to the variability in platelet recovery after *H. pylori* eradication among different populations.

Table 1 Prevalence of *Helicobacter pylori* infection and platelet response after *Helicobacter pylori* eradication in children with immune thrombocytopenia *n* (%)

Ref.	Country	Prevalence of *Helicobacter pylori* infection	Platelet response
Rajantie *et al*[29]	Finland	0/17 (0)	ND
Jaing *et al*[30]	Taiwan	9/22 (41)	5/9 (50)
Hayashi *et al*[31]	Japan	2/10 (20)	1/1 (100)
Yetgin *et al*[32]	Turkey	11/35 (31)	0/9 (0)
Jaing *et al*[33]	Taiwan	10/63 (16)	ND
Loffredo *et al*[34]	Italy	8/39 (21)	0/8 (0)
Neefjes *et al*[35]	Netherlands	3/47 (6)	3/3 (100)
Wu *et al*[36]	Taiwan	6/32 (19)	ND
Bisogno *et al*[37]	Italy	8/24 (33)	1/8 (13)
Hamidieh *et al*[38]	Iran	4/31 (13)	0/4 (0)
Treepongkaruna *et al*[39]	Thailand	16/55 (29)	0/7 (0)
Ferrara *et al*[40]	Italy	8/24 (33)	8/8 (100)
Maghbool *et al*[41]	Iran	5/30 (17)	5/5 (100)
Russo *et al*[42]	Italy	50/244 (20)	13/33 (39)

ND: Not described.

Childhood ITP

The clinical course of ITP is quite different in children than in adult patients. ITP in children is usually an acute form with spontaneous recovery within 6 mo, although thrombocytopenia lasts more than 6 mo in about 20% of children with ITP[28]. There are only a few pediatric studies evaluating the role of *H. pylori* infection in chronic ITP in children. Table 1 summarizes the studies assessing the prevalence of *H. pylori* infection and platelet response after eradicating *H. pylori* in children with ITP. Although a Finnish study failed to detect *H. pylori* infection in any of 17 children with chronic ITP[29], other studies detected *H. pylori* infection in a small proportion of children with chronic ITP[30-42]. The highest prevalence was reported in a study conducted in Taiwan, which showed an infection rate of 41%[29]. A recent multicenter study in Italy revealed that 50 (20) of 244 children with ITP were in-

fected with *H. pylori*[42]. In general, the prevalence of *H. pylori* infection is lower in children than in adults with ITP in a given population.

Reports of platelet recovery after *H. pylori* eradication therapy in children are highly variable and inconsistent. In the first study in Taiwan, platelet counts increased in 5 (56) of 9 *H. pylori*-infected children with ITP after eradication therapy[29]. In studies from the Netherland, Italy, and Iran, platelet counts increased measurably in all *H. pylori*-infected children after eradication treatment, although the number of subjects analyzed was very small (3, 8, and 5, respectively)[35,40,41]. However, other studies from Turkey, Italy, Iran, and Thailand showed that none of the patients who underwent treatment to eradicate *H. pylori* infection responded to it[32,34,38,39]. Even studies from the same country gave diametrically opposite accounts of the efficacy of *H. pylori* eradication. These contradictory results may be partly due to the small number of *H. pylori*-infected children with ITP. An Italian study, which had the largest number of patients, showed that 13 (39) of 33 children with *H. pylori* infection responded to the eradication treatment[42]. The prevalence of *H. pylori* infection in children with chronic ITP is generally low, suggesting that *H. pylori* infection plays only a minor role in the development of childhood ITP. However, we should recognize that some children infected with *H. pylori* can recover if the *H. pylori* is successfully eliminated. A large-scale study is necessary to confirm the relationship between *H. pylori* infection and childhood ITP.

Other forms of secondary ITP

ITP may develop in the context of other disorders or conditions, including lymphoproliferative, autoimmune, and infectious diseases[43]. Although *H. pylori*-associated ITP is now categorized as a secondary ITP condition, the efficacy of *H. pylori* eradication therapy has not been deeply examined in patients with other types of secondary ITP. To evaluate the role of *H. pylori* infection in the pathogenesis of secondary ITP, we conducted an open-label prospective study involving 34 consecutive *H. pylori*-infected patients with ITP, including 16 with primary ITP, 8 with secondary ITP associated with SLE, and 10 with secondary ITP associated with liver cirrhosis. All the patients received a standard *H. pylori* eradication regimen consisting of amoxicillin, clarithromycin, and lansoprazole, and the *H. pylori* was successfully eradicated in all except one patient with primary ITP, and another with liver cirrhosis. As shown in Figure 1, the platelet count had increased 3 mo after eradication treatment in nearly all the patients with primary ITP, but was virtually unchanged in patients with SLE or liver cirrhosis. In addition, a decrease in circulating anti-GP Ⅱb/Ⅲa antibody-producing B cells was observed in patients with primary ITP, but not in ITP patients with SLE or liver cirrhosis. These findings clearly indicate that the eradication of *H. pylori* fails to improve the pathogenic process in patients with some forms of secondary ITP. Thus, the efficacy of *H. pylori* eradication therapy is likely to be restricted to patients in a subgroup of seemingly primary ITP (*H. pylori*-associated ITP), and *H. pylori* infection appears to be uninvolved in the pathogenic process of other secondary ITPs.

CHARACTERISTICS OF *H. PYLORI*-ASSOCIATED ITP

Features of ITP patients infected with H. pylori

Several authors have tried to identify characteristics of *H. pylori*-associated ITP by comparing the clinical features of adult ITP patients with or without *H. pylori* infection. ITP patients infected with *H. pylori* were significantly older than uninfected patients[9], but this is predictable because the prevalence of *H. pylori* infection increases with age in the general population[44]. Multiple studies have failed to detect significant differences in any other demographic or clinical characteristic, including sex, platelet count, or response to therapy.

Several studies have looked for differences in genetic factors in ITP patients with or without *H. pylori*. Veneri *et al*[45] examined the human leukocyte antigen (HLA)-DRB1 and *DQB1* alleles in Italian patients with ITP, and found that *H. pylori*-positive patients had a lower frequency of *DRB1*03*, and higher frequencies of *DRB1*11*, *DRB1*14*, and *DQB1*03*, compared with *H. pylori*-negative patients. However, we failed to detect any association between *H. pylori* infection and HLA-DRB1 or DQB1 alleles in Japanese patients with ITP[46]. Instead, we found that gene polymorphism within the loci for interleukin (IL)-1β was associated with *H. pylori* infection in patients diagnosed before age 50. While these observations suggest an involvement of genetic background in *H. pylori*-related ITP, the associations should be confirmed by replication studies enrolling patients from other populations.

On the other hand, there was no difference in the IL-2, IL-4, or IL-6 serum levels between patients with and without *H. pylori* infection[47]. The serum levels of chemokines, including monocyte chemoattractant protein-1, regulated upon activation normally T-cell expressed and secreted, and epithelial cell-derived neutrophil attractant-78, were significantly higher in patients with *H. pylori* infection than in those without[48], although increased levels of these chemokines were also observed in individuals that had *H. pylori*-related gastrointestinal disorders but did not have ITP. Thus, studies have failed to identify demographic, clinical, genetic, or immunologic characteristics unique to ITP patients infected with *H. pylori*. This is probably because there are at least two distinct subgroups of *H. pylori*-infected ITP patients: those with *H. pylori*-associated secondary ITP, who respond to eradication therapy, and those with primary ITP and a coincidental *H. pylori* infection.

Factors predicting a positive response to H. pylori eradication therapy

Parameters that predict the platelet response to *H. pylori* eradication therapy have been extensively analyzed in

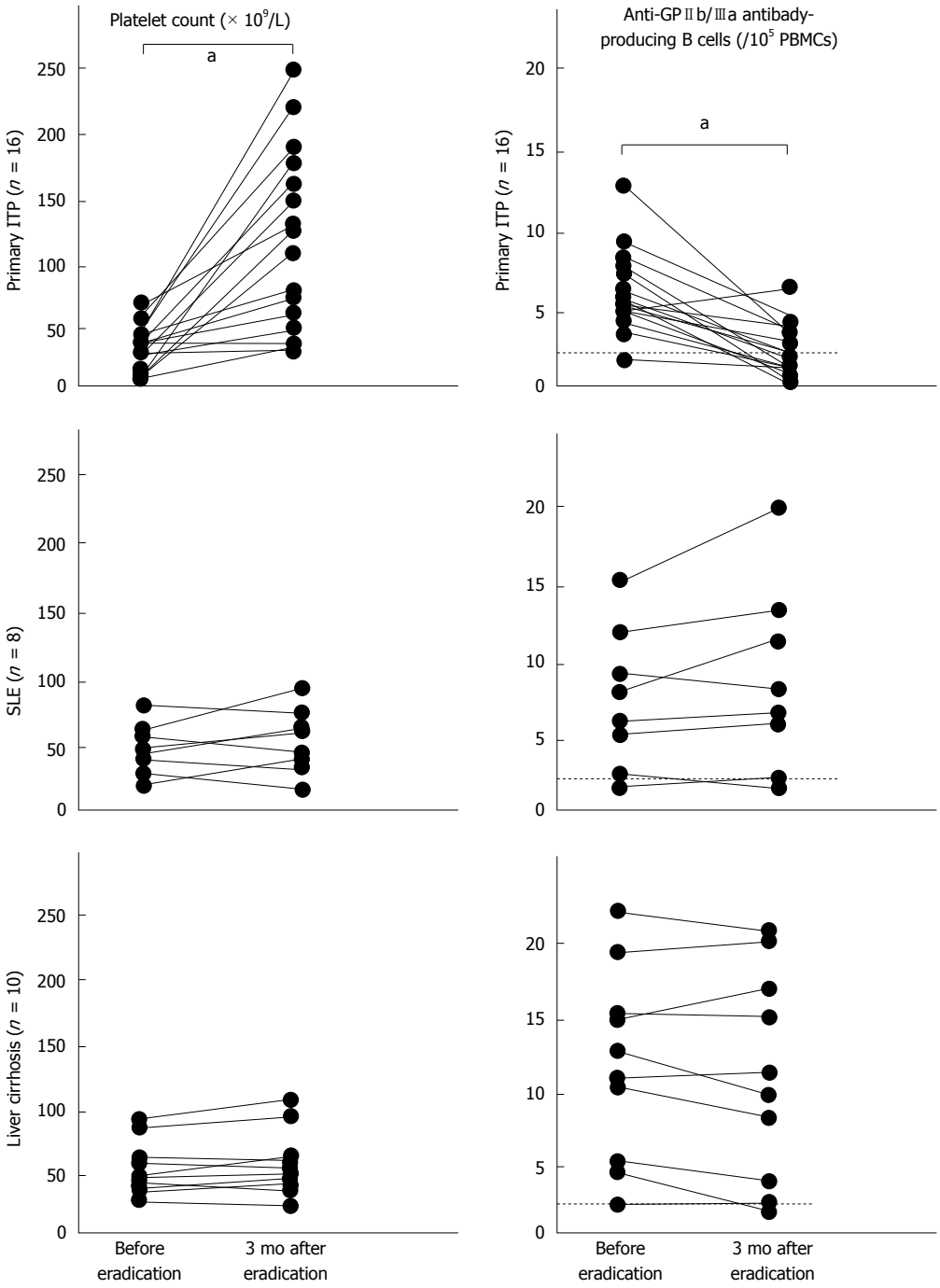

Figure 1 Changes in platelet count and in anti-GPⅡb/Ⅲa antibody-producing circulating B cells before and 3 mo after an *Helicobacter pylori* eradication regimen in *Helicobacter pylori*-positive immune thrombocytopenia patients with no additional disease, or with systemic lupus erythematosus or liver cirrhosis. Changes in absolute values were compared by paired *t* test. $^{a}P < 0.05$. A dotted line indicates the cut-off for circulating anti-GPⅡb/Ⅲa antibody-producing B cells, which was 2/10^{5} peripheral blood mononuclear cells (PBMCs). ITP: Immune thrombocytopenia; SLE: Systemic lupus erythematosus.

H. pylori-infected ITP patients. The most consistently reported feature that predicts a favorable response is a shorter duration of ITP[9,49,50], but other studies have not found this association[51-53]. Other clinical characteristics, including an age less than 65 when diagnosed with ITP[49], a higher baseline platelet count[49], no prior corticosteroid therapy[51], no concomitant corticosteroid therapy[54,55], and no prior therapy for ITP[49], have been reported as factors predicting the platelet response, but other studies have reported conflicting results.

Several studies have examined whether there is a genetic predisposition to the platelet response. An association was shown between the *HLA-DQB1*03* haplotypes and a higher probability of the platelet response[45]. Moreover, single nucleotide polymorphisms within the genes for tumor necrosis factor-β and an inhibitory Fcγ receptor ⅡB (FcγRⅡB) were found to be useful for predicting the response to the eradication treatment[55,56]. In terms of the anti-platelet autoantibody specificity, we found that the presence of an anti-GPIb autoantibody

response predicts resistance to *H. pylori* eradication therapy. Interestingly, a study from Italy reported that ITP patients with antibodies to CagA were more likely to respond to eradication therapy than patients without these antibodies[57]; although a study conducted in Japan failed to confirm this observation[50], we should recognize that CagA-positive strains are more common in Japan than in Italy.

Finally, Sato *et al*[58] assessed potential associations of the platelet response to *H. pylori* eradication with upper gastrointestinal endoscopic findings and histologic features of stomach tissue obtained by biopsy. A severe degree of gastric atrophy on endoscopy and intense inflammation and atrophy in the gastric corpus detected by biopsy were predictors for a favorable response. These findings together indicate that both genetic background and bacterial factors, which collaboratively regulate the host inflammatory response to the bacterium, can account, at least in part, for the variable response to *H. pylori* eradication therapy.

MECHANISMS OF *H. PYLORI*-ASSOCIATED ITP

It has become clear that *H. pylori*-associated ITP is a subset of ITP in which *H. pylori* infection is actively involved in the pathogenic process. In patients with *H. pylori*-associated ITP, *H. pylori* eradication increases the platelet count in parallel with a suppression of anti-platelet autoantibody production, and results in the remission or even cure of the disease in many patients. Since eradicating *H. pylori* does not increase the platelet count in non-ITP subjects[59], the platelet recovery after successful *H. pylori* eradication is specific to ITP patients, and is likely to be mediated through the inhibition of an ongoing autoimmune response to platelets.

Molecular mimicry

Several hypotheses have been proposed regarding the mechanism by which *H. pylori* induces the development of ITP. One intriguing theory is that cross-reactive antibodies are produced that react with both *H. pylori* components and platelet surface antigens through molecular mimicry. Michel *et al*[22] investigated this molecular-mimicry hypothesis by testing platelet eluates derived from *H. pylori*-infected ITP patients, and found that platelet eluates with the capacity to react with GP Ⅱb/Ⅲa or GPIb failed to recognize any *H. pylori* antigens. On the other hand, Takahashi *et al*[25] reported that platelet eluates from *H. pylori*-infected ITP patients recognized CagA in immunoblots, but those from *H. pylori*-infected non-thrombocytopenic individuals did not. Unfortunately, since the IgG concentrations in the eluates were not adjusted in these studies, the intensity of individual bands was not quantitative. In addition, since platelets are known to take up and concentrate circulating IgG in intracellular granules, it is not clear whether the anti-CagA antibodies detected truly cross-react with platelet-surface antigens. Although it was recently reported that monoclonal antibodies generated against recombinant *H. pylori* urease B react with GP Ⅱb/Ⅲa expressed on the platelet surface, this study failed to show the presence of this cross-reactive antibody repertoire in patients with *H. pylori*-associated ITP[60]. While these findings suggest that cross-reacting antibodies against *H. pylori* may be present in patients with ITP, their pathogenic role remains obscure.

Non-specific activation of the immune system

In another potential mechanism, chronic *H. pylori* infection may act on the host's immune system to stimulate acquired immune responses, causing autoreactive T and B cells to emerge. Yamanishi *et al*[61] showed that *H. pylori* components are able to initiate autoimmune responses *via* autoantibodies that are produced through the activation of B-1 cells. However, this non-specific mechanism alone does not explain how an autoimmune response specific to platelet glycoproteins, such as that observed in ITP patients, would develop. In fact, there is no difference in the production of non-specific autoantibodies, including anti-nuclear, anti-microsome, and anti-smooth muscle antibodies, in individuals with and without *H. pylori*[62].

Modulation of monocyte/macrophage function

We have been evaluating mechanisms that elicit and maintain autoantibody responses to platelet glycoproteins in patients with ITP[63-65]. After detailed analyses of the GP Ⅱb/Ⅲa-reactive $CD4^+$ T cells and B cells in ITP patients, we proposed a "pathogenic loop" model for the ongoing IgG anti-platelet autoantibody response in ITP patients[66]. Specifically, macrophages in the reticuloendothelial system capture opsonized platelets *via* Fcγ receptors, and present antigenic platelet glycoprotein-derived peptides to T cells. Autoreactive $CD4^+$ T cells are then activated by their recognition of the antigenic peptides and exert helper activity to stimulate B cells to produce IgG anti-platelet autoantibodies, which in turn bind to circulating platelets. Theoretically, once this pathogenic loop is established, the production of IgG anti-platelet autoantibodies continues endlessly. Since anti-platelet autoantibodies are eliminated after eradicating *H. pylori* in patients with *H. pylori*-associated ITP[12], this pathogenic loop would consequently be disrupted and blocked. To elucidate the mechanism by which *H. pylori* eradication changes the ongoing pathogenic loop in ITP patients, we conducted a prospective study in which the phenotype and function of the autoreactive T and B cells and of the monocytes/macrophages involved in the pathogenic loop were serially measured in *H. pylori*-infected and -uninfected ITP patients treated with a standard eradication regimen[67]. At baseline, we found enhanced phagocytic capacity and low expression levels of inhibitory FcγR Ⅱ B in the circulating monocytes from *H. pylori*-infected patients, but not in those from uninfected patients. Suppression of this activated-monocyte phenotype was observed one week after starting the *H. pylori* eradication regimen,

when eradication was successful. The anti-platelet autoantibody responses and platelet kinetic parameters subsequently improved, indicating that suppression of the activated-monocyte function precedes the improvement in the autoantibody response.

Interestingly, modulation of the Fcγ-receptor balance toward an activating phenotype has also been observed in *H. pylori*-infected mice, through a downregulation of inhibitory FcγR Ⅱ B in splenic and circulating monocytes/macrophages. A study in China recently confirmed that the FcγR Ⅱ B expression on circulating monocytes is down-regulated in *H. pylori*-infected ITP patients[68]. Therefore, *H. pylori* infection plays an important role in ITP pathogenesis by altering the Fcγ receptor balance of monocytes/macrophages in favor of activating Fcγ receptors, through downregulation of the inhibitory receptor FcγR Ⅱ B. These findings indicate that the platelet recovery after *H. pylori* eradication in ITP patients is mediated, at least in part, through a change in Fcγ receptor balance toward the inhibitory FcγR Ⅱ B. The molecular events that induce this change in monocytes/macrophage properties are unclear. Although *H. pylori* does not invade the gastric epithelium, it can induce both the secretion of soluble inflammatory mediators and cellular apoptosis in the host, leading to local inflammation in the epithelium and the subepithelial layers. Released *H. pylori* components have been reported to be responsible for activating dendritic cells and macrophages through toll-like receptor signaling[69,70].

Interestingly, a change in Fcγ receptor balance toward the inhibitory FcγR Ⅱ B in monocytes/macrophages has also reported in the therapeutic action of other established treatment regimens for ITP, such as intravenous immunoglobulin and high-dose dexamethasone. Samuelsson et al. demonstrated that intravenous immunoglobulin requires the presence of FcγR Ⅱ B to prevent antibody-induced thrombocytopenia in a murine model of passive ITP[71], in which the FcγR Ⅱ B expression on splenic macrophages was upregulated upon intravenous immunoglobulin treatment. The upregulation of inhibitory FcγR Ⅱ B expression on circulating monocytes was also reported in ITP patients successfully treated with high-dose dexamethasone[72]. These findings point to the Fcγ receptor balance of monocytes/macrophages as an attractive therapeutic target for ITP.

A multifactorial mechanism

The pathogenesis of *H. pylori*-associated ITP most likely involves several factors. The mechanism of Fcγ-receptor-balance modulation in monocytes/macrophages does not exclude other proposed mechanisms for platelet recovery in ITP after *H. pylori* eradication, such as molecular mimicry between CagA and platelet-surface antigens. Moreover, some *H. pylori* strains induce platelet aggregation that is dependent on the interaction of von Willebrand factor and IgG antibodies against *H. pylori* with their corresponding receptors, GPIb and FcγR Ⅱ A, on platelets[73]. In this model, anti-*H. pylori* antibodies are capable of opsonizing platelets by binding to *H. pylori*, von Willebrand factor, and GP Ⅰ b, like anti-platelet autoantibodies. However, we should recognize that *H. pylori* infection is usually established in infants with an immature immune system, and that the prevalence of ITP among *H. pylori*-infected individuals is extremely low. Therefore, it is apparent that *H. pylori* infection alone is insufficient to induce the onset of ITP. Additional triggers would be necessary to elicit the anti-platelet autoimmune response observed in *H. pylori*-associated ITP.

CONCLUSION

The past few years have witnessed important advances in our understanding of the relationship between *H. pylori* infection and ITP. The sustained efficacy of the *H. pylori* eradication regimen in adults with ITP has affected decision-making in clinical practice. The importance of detecting and eradicating *H. pylori* in patients with seemingly typical ITP is now recognized, especially where *H. pylori* infection is prevalent, such as in Japan. A growing body of evidence indicates that *H. pylori*-associated ITP is a unique ITP subset in which the bacterium is central to the pathogenic process. The development of *H. pylori*-associated ITP appears to depend on multiple factors. Among these, modulation of the Fcγ receptor balance of monocytes/macrophages through inhibition of the immunosuppressive FcγR Ⅱ B signal, which is a host immune response associated with *H. pylori* infection, is a key mechanism for initiating and maintaining the anti-platelet autoantibody response. These insights provide valuable clues that may assist in the development of new therapeutic strategies for ITP.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (6): *Helicobacter pylori*

Efficacy of fermented milk and whey proteins in *Helicobacter pylori* eradication: A review

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Abstract

Helicobacter pylori (*H. pylori*) eradication is considered a necessary step in the management of peptic ulcer disease, chronic gastritis, gastric adenocarcinoma and mucosa associated lymphoid tissue lymphoma. Standard triple therapy eradication regimens are inconvenient and achieve unpredictable and often poor results. Eradication rates are decreasing over time with increase in antibiotic resistance. Fermented milk and several of its component whey proteins have emerged as candidates for complementary therapy. In this context the current review seeks to summarize the current evidence available on their role in *H. pylori* eradication. Pertinent narrative/systematic reviews, clinical trials and laboratory studies on individual components including fermented milk, yogurt, whey proteins, lactoferrin, α-lactalbumin (α-LA), glycomacropeptide and immunoglobulin were comprehensively searched and retrieved from Medline, Embase, Scopus, Cochrane Controlled Trials Register and abstracts/proceedings of conferences up to May 2013. A preponderance of the evidence available on fermented milk-based probiotic preparations and bovine lactoferrin suggests a beneficial effect in Helicobacter eradication. Evidence for α-LA and immunoglobulins is promising while that for glycomacropeptide is preliminary and requires substantiation. The magnitude of the potential benefit documented so far is small and the precise clinical settings are ill defined. This restricts the potential use of this group as a complementary therapy in a nutraceutical setting hinging on better patient acceptability/compliance. Further work is necessary to identify the optimal substrate, fermentation process, dose and the ideal clinical setting (prevention/treatment, first line therapy/recurrence, symptomatic/asymptomatic, gastritis/ulcer diseases *etc.*). The potential of this group in high antibiotic resistance or treatment failure settings presents interesting possibilities and deserves further exploration.

Key words: *Helicobacter pylori*; Fermented milk; Whey proteins; Bovine lactoferrin; α-Lactalbumin; Glycomacropeptide; Immunoglobulin

Core tip: Treatment regimens for *Helicobacter* are cumbersome, prone to side effects and often have low success rates. Fermented milk and related proteins have often been explored as potential candidates for complementary therapy. The current review sought to summarize the current evidence available on their role in *Helicobacter pylori* eradication and found substantial evidence to support the use of fermented milk based probiotic preparation and bovine lactoferrin. Evidence for other whey proteins is preliminary and requires substantiation. Further work is necessary to identify the optimal substrate, fermentation process, dose and

the ideal clinical setting. The potential of this group in antibiotic resistance or treatment failure settings also presents interesting possibilities.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a gram negative, spiral shaped bacterium found in the gastric mucous layer. It has an ammonia-producing surface urease which allows adherence to and colonization of the gastric epithelium, by neutralizing the acidic gastric environment[1]. *H. pylori* is now implicated in peptic ulcer disease, chronic gastritis, gastric adenocarcinoma, mucosa associated lymphoid tissue lymphoma and duodenal ulcer disease[2-4]. Eradication of *H. pylori* is considered a necessary step in the management of these diseases. Standard triple therapy eradication regimens (proton pump inhibitor plus clarithromycin and amoxicillin or nitroimidazole) are inconvenient and achieve unpredictable and often poor results[5]. Further, eradication rates are reported to be decreasing over time with an increase in antibiotic resistance[6]. Second line quadruple regimens are further limited by poorer patient compliance and increased side effects[6]. In this context, several alternative and complementary therapies have been tried in an attempt to achieve better eradication without affecting compliance. In this search, fermented milk and several of its component whey proteins have emerged as potential candidates for complementary therapy. They have the inherent advantage of better patient acceptability.

Several randomized controlled trials and a recent meta-analysis document that fermented milk-based probiotic preparations improve *H. pylori* eradication rates by 10%. Their efficacy has been argued to be better than capsule-based bacteria-only preparations and considered partly or completely contributed by the anti-bacterial and immunogenic properties of component whey proteins formed as a result of fermentation *etc*. Potential efficacy of individual whey proteins in *H. pylori* eradication has also been a subject of interest in recent research. However the role of fermented milk or whey proteins in clinical practice is not yet universally accepted, precisely defined or widely discussed[7]. In this context the current review sought to summarize the current evidence available on the role of fermented milk and its component whey proteins in *H. pylori* eradication.

For the purpose of the current review pertinent narrative/systematic reviews, clinical trials and laboratory studies on individual components including fermented milk, yogurt, whey proteins, lactoferrin, α-lactalbumin (α-LA), glycomacropeptide and immunoglobulin were comprehensively searched and retrieved from Medline, Embase, Scopus, Cochrane Controlled Trials Register and abstracts/proceedings of conferences up to May 2013. The available studies/meta-analysis were rated for quality as per the Scottish Intercollegiate Guidelines Network (SIGN) check lists[8] and the Quality Rating for Individual Studies[9]. The evidence was subsequently graded using the Revised Grading System[10]. The level of recommendation was later defined into one of four grades (A, B, C or D; SIGN grades)[11].

FERMENTED MILK

Fermented milk refers to whole or skimmed milk curdled to a beverage or custard like consistency by lactic acid producing bacteria. A wide assortment of products, varying by the process, bacteria, duration and other variables, are available and widely consumed in different countries. However, there are several commonalities. Fermented milk possesses a protein system constituted by two major families of proteins *i.e.*, casein and whey proteins. Casein is insoluble, and accounts for 80% of the whole protein inventory. Whey proteins are globular water soluble molecules and include bovine lactoferrin, α-LA, glycomacropeptide, immunoglobulin, β-lactoglobulin and lactoperoxidase. Whey is thought to have the ability to act as an antioxidant, immune enhancer, antihypertensive, antitumor, hypolipidemic, antiviral, antibacterial and as a chelating agent[12].

In the context of Helicobacter eradication there is a fair body of evidence from trials conducted using fermented milk (usual culturally/commercially available preparations including yogurt), fermented milk based probiotic preparations (FMPPs; fermented milk with specifically added live probiotic bacteria like Lactobacilli) and capsule based probiotics. An observational study on 464 healthy Mexican subjects documented lower prevalence of *H. pylori* seropositivity in those consuming yogurt more than once a week compared with non-consumers[13]. As presented in Table 1, several clinical trials and a systematic review of RCTs compared an FMPP *vs* placebo or standard therapy plus FMPP *vs* standard therapy and documented a beneficial effect of FMPPs[14]. The overall quality and quantity of evidence for FMPPs appears convincing (Recommendation Grade-A) and beneficial effect appears to be sustained when FMPP were used in combination with standard therapy (Recommendation Grade A[15-18]). Also, benefit has been documented in symptomatic children (Recommendation Grade-B), symptomatic and asymptomatic adults (Recommendation Grade-B) and in patients who failed eradication on standard therapy (Recommendation Grade-B). The overall magnitude of the benefit was estimated to be 5%-15%[14].

With reference to the active principle components responsible for this effect, the available clinical evidence can be better summarized on the basis of three argu-

Table 1 Studies comparing "fermented milk based probiotic preparation" with placebo or "standard therapy + fermented milk based probiotic preparation" with "standard therapy"

Ref.	Type of trial	Evidence grade[1]	Quality rating[2]	Subjects	Study design	Study groups/methods	Outcome variable/s	Results and conclusions
Positive								
Bekar *et al*[15], 2011, Turkey	Human	1+	+	82 pts of dyspepsia and *H. pylori* infection	RCT	Two groups - Control group (n = 36; Triple therapy - lansoprazole, clarithromycin and amoxicillin + placebo) and Treatment group [n = 46; Triple therapy + kefir (fermented milk drink containing probiotics)]; given for 14 d	Eradication of *H. pylori*; adverse events of eradication therapy (Urease test after 45 d of treatment)	Significantly more patients (78.2% *vs* 50.0%) in the treatment group achieved eradication in comparison with control group. Side effects were less frequent and less severe in the treatment group
Sachdeva *et al*[14] 2009, India	Metaanalysis	1+	++	10 eligible trials; data available for 963 patients	Meta-analysis of human RCTs/ CCTs	Trials had to be randomized or quasi-randomized and controlled, using a FMPP in the intervention group treating *Helicobacter*-infected patients. The only difference between the two groups had to be FMPP	Eradication of *H. pylori*; adverse events of eradication therapy	The pooled odds ratio for eradication by ITT analysis in the treatment *vs* control group was 1.91 (1.38-2.67; P < 0.0001) using fixed effect model The pooled risk difference was 0.10 (95%CI: 0.05-0.15; P < 0.0001) by fixed effect model. Fermented milk based probiotic preparations improve *H. pylori* eradication rates by approximately 5%-15%, whereas the effect on adverse effects is heterogeneous
Sýkora *et al*[16], 2005, Czech Republic and United Kingdom	Human	1+	++	86 symptomatic *H. pylori* positive children	RCT	Two groups - OAC-LC group - Omeprazole, amoxicillin and clarithromycin for 7 d with fermented milk containing *L. casei* DN-114001 for 14 d (n = 39) *vs* OAC group - Omeprazole, amoxicillin and clarithromycin for 7 d (n = 47)	Eradication of *H. pylori*, Endoscopic and Histologic comparison	ITT based eradication rates for the group A were 84.6% and 91.6% by PP analysis. Eradication in the group B was 57.5% in the ITT and 61.3% in the PP group. Eradication success was higher in the group A compared to group B in both ITT (P = 0.0045) and PP analysis (P = 0.0019)
Sheu *et al*[17], 2006, Taiwan	Human	1+	+	138 patients in whom triple therapy failed	RCT	Two groups - yogurt (containing *L. acidophilus La5, Lactobacillus bulgaricus, Bifidobacterium lactis Bb12* and *Streptococcus thermophilus*)-plus-quadruple therapy group for 7 d (n = 69) *vs* quadruple therapy only group (n = 69) for 7 d	Successful eradication of *H. pylori*, drug compliance, side effects	The yogurt-plus-quadruple therapy group had a higher *H. pylori* eradication rate than did the quadruple therapy only group (ITT analysis 85% *vs* 71.1%, P < 0.05; PP analysis- 90.8% *vs* 76.6%, P < 0.05). Side effects were more frequent in the quadruple therapy-only group than in the yogurt-plus-quadruple therapy group
Miki *et al*[20], 2007, Japan	Human	1-	++	69 subjects who were positive for *H. pylori* infection	RCT	Two groups - Fermented milk (*Bifidobacterium bifidum YIT*) (BF-1) (n = 34) *vs* placebo (untreated milk) (n = 35) for 12 wk	Suppressive effect of BF-1 fermented milk on *H. pylori* urease activity and gastric situation	*H. pylori* infection was judged by the C-UBT. *H. pylori*-negativity (below 5%: n = 6 and 4 in the BF-1 and placebo groups, respectively) subjects
Sheu *et al*[18], 2002, Taiwan	Human	1-	+	160 *H. pylori* infected patients	CCT	Two groups - triple plus yogurt (TYG) (containing *L. acidophilus La5, Lactobacillus bulgaricus, Bifidobacterium lactis* Bb12 and *Streptococcus thermophilus*) group (n = 80) *vs* triple only group (TG) (n = 80) for 7 d	Successful eradication of *H. pylori*, drug compliance, side effects	By ITT analysis, the triple-plus-yogurt group had a higher *H. pylori* eradication rate than the triple-only group (P < 0.05) and side effects were more commonly found in the TG than in the TYG. Also a significantly higher proportion of patients in the TYG completed the 7-d regimen than in the TG (67.5% *vs* 43.8%, P < 0.05)
Felley *et al*[21], 2001, Boston	Human	1-	+	53 volunteers infected with *H. pylori*	CCT	Two groups - Acidified milk containing *L. johnsonii* La1 (LC-1) (n = 25) *vs* Placebo (pasteurized milk) (n = 27) for 3 wk followed by 500 mg bid clarithromycin received by all subjects during the last 2 wk	Effect of the given treatment on *H. pylori* density, gastric inflammation and activity	In the LC-1 group, four had higher scores in the antrum, 14 were found to have a decreased *H. pylori* density reflected by lower scores (P = 0.02) and in the placebo group in antrum scores remain identical in 10 volunteers and decreased in 11 (0.08). The results suggest that *H. pylori* infection and gastritis can be down-regulated by LC-1

Cats *et al*[22], 2003, Netherlands	Human	1-	-	14 *H. pylori* positive subjects	CCT	Two groups - Fermented milk (*L.casei*) for 3 wk (n = 14) *vs* control group (n = 6)	Effect of *L.casei* on urease activity *in vivo* (*H. pylori* positive subjects)	Urease activity decreased in nine of the 14 (64%) subjects with *L. casei* supplementation and in two of the six (33%) controls (P = 0.22). A slight, but non-significant, trend towards a suppressive effect of *L. casei* on *H. pylori* *in vivo* may exist
Wang *et al*[19], 2004, Taiwan	Human	1-	-	70 volunteers infected with *H. pylori*	CCT	Two groups - AB yogurt (*containing L. acidophilus* La5, *Lactobacillus bulgaricus*, *Bifidobacterium lactis* Bb12 and *Streptococcus thermophilus*) (n = 59) *vs* milk placebo (n = 11) for 6 wk	Effect of yogurt on *H. pylori* infection in humans	Administration of AB-yogurt decreased the urease activity of *H. pylori* after 6 wk of therapy (P < 0.0001). Regular intake of yogurt containing Bb12 and La5 effectively suppressed *H. pylori* infections in humans
Park *et al*[23], 2001, South Korea	Human	NR	-	40 *H. pylori* infected volunteers	CCT	Two groups - Fermented milk (*Lactobacillus acidophilus*, *Lactobacilus casei*) (n = 21) *vs* Placebo (n = 19) for 4 wk	Eradication of *H. pylori*, Comparison of endoscopic findings, Compliance	All patients were compliant and the *H. pylori* density of antrum tended to decrease in treatment group compared with placebo group (P = 0.072). 3 cases in treatment group were noted for negative conversions of both rapid urease test and C-UBT
Kim *et al*[24], 2007, South Korea	Human	FTNA	FTNA	262 *H. pylori* infected patients	CCT	Two groups - triple plus yogurt group for 3 wk (n = 147) *vs* triple only group (n = 115) for 1 wk	Eradication of *H. pylori*	In PP analysis, *H. pylori* eradication rate in the yogurt group, 87.7% was marginally higher than that in control group, 78.4% (P = 0.055). And according to ITT analysis, the eradication rate in the yogurt group, 78.2% was also marginally higher than that of control group, 69.5% (P = 0.062)
Negative								
Goldman *et al*[25], 2006, Argentina	Human	1+	++	65 children who tested positive for *H. pylori*	RCT	Two groups - triple therapy with probiotic food (commercial yogurt containing *Bifidobacterium animalis* and *Lactobacillus casei*) (n = 33) *vs* triple therapy with placebo (milk fluid) (n = 32)	Eradication of *H. pylori*	We found no significant differences in *H. pylori* eradication rates at 1 and 3 mo between the treated group (ER 45.5% and 42.4%) and the control group (ER = 37.5% and 40.6%). Study could not demonstrate an adjuvant effect of the studied probiotic food to triple therapy in the eradication of *H. pylori* infection in children
Song *et al*[26], 2005, South Korea	Human	NA	-	70 patients with duodenal ulcer	CCT	Two groups - triple-plus-fermented milk (*Lactobacilli*) (n = 35) *vs* triple plus placebo (n = 35)	*H. pylori* eradication rate, Fermented milk group reduces treatment-related adverse reactions	Eradication was successful in 88.6% in the *Lactobacilli* group and 85.7% in the placebo group (P = 1.00). *Lactobacillus* containing fermented milk couldn't exert beneficial effects on *H. pylori* eradication or treatment-related adverse reactions

[1]Levels of evidence: 1++ High quality meta-analysis, systematic reviews of RCTs, or RCTs with a very low risk of bias; 1+ Well conducted meta-analysis, systematic reviews of RCTs or RCTs with a low risk of bias; 1- Meta-analysis, systematic reviews or RCTs or RCTs with a high risk of bias; 2++ High quality systematic reviews of case-control or cohort studies or high quality case-control or cohort studies with a very low risk of confounding, bias, or chance and a high probability that the relationship is causal; 2+ Well conducted case control or cohort studies with a low risk of confounding, bias, or chance and a significant risk that the relationship is not causal; 3 Non-analytic studies, *e.g.*, case reports, case series; 4 Expert opinion. [2]Quality rating for individual studies: ++ Applies if all or most criteria from the checklist are fulfilled; where criteria are not fulfilled the conclusions of the study or review are thought very unlikely to alter; + Applies if some of the criteria from the checklist are fulfilled; where criteria are not fulfilled or are not adequately described, the conclusions of the study or review are thought unlikely to alter; - Applies if few or no criteria from the checklist are fulfilled; where criteria are not fulfilled or are not adequately described, the conclusions of the study or review are thought likely or very likely to alter. *H. pylori*: *Helicobacter pylori*; RCT: Randomised controlled trial; CCT: Controlled clinical trial; CT: Clinical trial; C-UBT: ^{13}C-urea breath test; FMPP: Fermented milk based probiotic preparation; NR: Not reported; FTNA: Full text not available; NS: Not significant; LC-1: *L. johnsonii* La1.

ments (Tables 1, 2 and 3). First, if whey proteins have clinically significant anti-Helicobacter properties then FMPP alone or in combination with standard therapy should have documented effectiveness (improvement in eradication rates)[15-26]. Secondly, capsule based probiotic preparations (bacteria only) should be partly or completely ineffective in *H. pylori* eradication[27-38]. Thirdly, if FMPP's are compared with a fermented milk control group then in the control group there should be some improvement partly or completely negating the effect of the addition of bacteria in the treatment group[39-41].

As summarized in Tables 1-3, the available evidence supports the above assertions and arguments. It is evident from the clinical studies and meta-analysis presented in Tables 1-3 that FMPPs have some efficacy against *Helicobacter* (10 positive trials and one positive meta-anal-

Table 2 Studies comparing capsule based probiotic (bacteria only) with placebo or standard therapy plus capsule based probiotic *vs* standard therapy

Ref.	Type of trial	Evidence grade[1]	Quality rating[2]	Subjects	Study design	Study groups/ methods	Outcome variable/s	Results and conclusions
Positive								
Canducci *et al*[27], Italy, 2000	Human	1+	+	120 *H. pylori* positive patients	RCT	Two groups: RCA (Rabeprazole, Clarithromycin, Amoxycillin) group- triple therapy (n = 60), RCAL group- triple therapy with Lactéol Fort for 7 d	Effect of *L. acidophilus* could improve the efficacy of a standard anti-*H. pylori* therapy	In RCA group eradication was successful in 72% at PP analysis or 70% at ITT analysis and in RCAL group eradication was achieved with 88% with PP analysis, 87% with ITT analysis
Negative								
Gotteland *et al*[28], 2005	Human	1+	+	254 children positive for *H. pylori*	RCT	Three groups: Antibiotics (group Ab)- (n = 57) for 8 d, *Lactobacillus acidophilus* LB (group Ab)- (n = 63) for 8 wk, *Saccharomyces boulardii* plus inulin (group Sb1)- (n = 62) 8 wk	To evaluate the capacity of *Lactobacillus acidophilus* LB and of symbiotic combination of Sb plus inulin to interfere with *H. pylori* colonization in children	*H. pylori* was eradicated in 66%, 12% and 6.5% of the children from the Ab, Sb1 and LB groups, respectively. A moderate but significant difference in Δ DOB was detected in children receiving living Sb1, but not in those receiving LB
Lionetti *et al*[29], 2006, Italy	Human	1+	++	40 *H. pylori* positive children	RCT	Two groups: Group A- 10 d sequential therapy plus *L. reuteri* ATCC 55730, Group B-Placebo with the same therapy	Effect of *Lactobacillus reuteri* to prevent or minimize the gastrointestinal side-effects	No significant differences were observed between the groups in the success of *H. pylori* eradication. Treatment was successful in 17 of 20 [85% (95%CI: 68-100)] patients in probiotic supplemented when compared with 16 of 20 patients in placebo group [80% (95%CI: 61–99)] (P = NS)
Nista *et al*[30], 2004, Italy	Human	1+	++	106 *H. pylori* positive patients	RCT	Two groups: Group A- triple therapy for 7 d plus *Bacillus clausii* (probiotic) for 14 d starting from the first day of the treatment (n = 54) Group B- triple therapy plus placebo (n = 52)	Effect of probiotic on incidence and severity of antibiotic-associated side-effects during anti- *H. pylori* therapy and eradication was evaluated with means of ^{13}C-urea breath test	The *H. pylori* eradication rate was similar between *B. B. clausii* and placebo groups. In particular, ITT analysis has shown *H. pylori* was eradicated in 39 of 54 patients (72.2%) in the *B. clausii* group and in 37 of 52 patients (71.15%) in the placebo group. In PP population, *H. pylori* was eradicated in 39 of 50 patients (78%) in the B. clausii group and in 37 of 50 patients (74%) in the placebo group
Myllyluoma *et al*[31], 2005, Finland	Human	1+	+	47 subjects with *H. pylori* infection	CCT	Two groups: Group A –probiotic drink (n = 23), group B- Placebo (n = 24) during *H. pylori* eradication and for 3 wk following the treatment	Effect of probiotic therapy on symptoms associated with the recommended *H. pylori* eradication treatment. As a secondary endpoint to find out whether this therapy could improve the eradication rate	The *H. pylori* eradication rate was non-significantly higher in the group receiving probiotic therapy (91% *vs* 79%, P = 0.42)
Cindoruk *et al*[32], 2007, Turkey	Human	1+	+	124 patients with *H. pylori* infection	RCT	Two groups: Group A- triple therapy plus *S. boulardii*, Group B- triple therapy plus placebo for 14 d	Efficacy and safety of *S. boulardii* in the prevention of side effects and the eradication success of anti-*H. pylori* therapy	*H. pylori* eradication rate, although higher in the treatment group, was statistically similar in treatment and control groups: 71% (44/62) *vs* 59.7% (37/62), respectively (P > 0.05)

Armuzzi *et al*[33], 2001, Italy	Human	1+	+	60 healthy asymptomatic subjects screened positive for *H. pylori* infection	CCT	Two groups: Group A- triple therapy for 7 d plus *Lactobacillus* GG for 14 d during and the week after eradication therapy, Group B- triple therapy plus placebo	Effect of probiotic *Lactobacillus* GG to minimize or to prevent the occurrence of gastrointestinal side effects	*H. pylori* eradication rates in group A was 83.33% (25/30) and in group B was 80% (24/30). *H. pylori* eradication rate had no significant difference
Guo *et al*[34], China, 2004	Human	FT NA	FT NA	97 *H. pylori* positive symptomatic patients	CCT	Two groups: treatment group (triple therapy plus Bifid triple viable capsule containing *Bifidobacteria longum*, faecal streptococci, *Lactobacillus acidophilus*) (n = 47) control group: triple therapy (n = 50)	Efficacy of probiotic in the treatment of *H. pylori*	Eradication rate was 93.6% (44/47) in treatment group and 88% in control group (44/50). *H. pylori* eradication rate had no significant difference
Armuzzi *et al*[35], 2001, Italy	Human	FT NA	FT NA	120 healthy asymptomatic subjects screened positive for *H. pylori* infection	CCT	Two groups: Group A- triple therapy for 7 d plus *Lactobacillus* GG for 14 d during and the week after eradication therapy, Group B- triple therapy plus placebo	Effect of probiotic *Lactobacillus* GG to minimize or to prevent the occurrence of gastrointestinal side effects.	*H. pylori* eradication rates in group A was 80% (48/60) and in group B was 76.67% (46/60). *H. pylori* eradication rate had no significant difference
Cremonini *et al*[36], Italy, 2002	Human	FT NA	FT NA	85 *H. pylori* positive, asymptomatic patients	CCT	Four groups- received both during and for 7 d after a 1 wk-triple therapy Group Ⅰ- *Lactobacillus* GG (n = 21), group Ⅱ-*Saccharomyces* boulardii (n = 22), group Ⅲ-*lactobacillus* spp. And bifidobacteria (n = 21), group Ⅳ-placebo (n = 21)	Efficacy of probiotic in the eradication of *H. pylori* infection	The *H. pylori* eradication rate was almost identical between the probiotic and placebo groups
Tursi *et al*[37], 2004, Italy	Human	FT NA	FT NA	70 patients with persistent *H. pylori* infection	CCT	Two groups- group A- quadruple therapy plus bacteria *lactobacillus casei* subsp. casei DG or group B- quadruple therapy only	Effect of probiotic supplementation on the effectiveness and tolerability of a new second-line 10 d quadruple therapy	*H. pylori* was negative in 33/34 group A patients (PP: 97.05% ITT: 94.28%) and 30/32 Group B patients
Cao *et al*[38], China, 2005	Human	FT NA	FT NA	128 *H. pylori* positive symptomatic patients	CCT	Two groups: Group A -quadruple therapy plus *Clostridium butyricum* group B- quadruple therapy	Effect of treatment given in eradication of *H. pylori*	Eradication rates in group A 96.88% (62/64) and group B 92.19% (59/64) was not significantly different

[1]Levels of evidence: 1++ High quality meta-analysis, systematic reviews of RCTs, or RCTs with a very low risk of bias; 1+ Well conducted meta-analysis, systematic reviews of RCTs or RCTs with a low risk of bias; 1- Meta-analysis, systematic reviews or RCTs or RCTs with a high risk of bias; 2++ High quality systematic reviews of case-control or cohort studies or high quality case-control or cohort studies with a very low risk of confounding, bias, or chance and a high probability that the relationship is causal; 2+ Well conducted case control or cohort studoes with a low risk of confounding, bias, or chance and a significant risk that the relationship is not causal; 3 Non-analytic studies, *e.g.*, case reports, case series; 4 Expert opinion. [2]Quality rating for individual studies: ++ Applies if all or most criteria from the checklist are fulfilled; where criteria are not fulfilled the conclusions of the study or review are thought very unlikely to alter; + Applies if some of the criteria from the checklist are fulfilled; where criteria are not fulfilled or are not adequately described, the conclusions of the study or review are thought unlikely to alter; - Applies if few or no criteria from the checklist are fulfilled; where criteria are not fulfilled or are not adequately described, the conclusions of the study or review are thought likely or very likely to alter. *H. pylori*: *Helicobacter pylori;* RCT: Randomised controlled trial; CCT: Controlled clinical trial; CT: Clinical trial; NR: Not reported; NS: Not significant.

ysis compared with 2 negative trials; Argument 1 above). It is also apparent from Table 1-3 that studies using capsule-based probiotic preparations are predominantly negative (1 positive trial compared with 11 showing no benefit; and Argument 2). In support of Argument 3 the overall data on the beneficial effect of bacterial probiotic

Table 3 Clinical trials comparing fermented milk based probiotic preparations *vs* plain fermented milk

Ref.	Type of trial	Evidence grade[1]	Quality rating[2]	Subjects	Study design	Study groups/ methods	Outcome variable/s	Results and conclusions	
Positive									
Pantoflickova *et al*[39], 2003, Switzerland	Human	1-	++	50 *H. pylori* positive healthy volunteers	RCT	Two groups- fermented milk with LC (n = 25) *vs* fermented milk as Placebo (n = 25). Subjects took the treatment twice daily during the first 3 wk and once daily for the next 13 wk	Effect of LC[1] intake without antibiotics on *H. pylori* gastritis, *H. pylori* density	LC[1] intake had a favorable, albeit weak, effect on *H. pylori* associated gastritis, particularly in the antrum. Regular ingestion of fermented milk containing *L.johnsonii* may reduce the risk of developing disorders associated with high degrees of gastric inflammation and mucus depletion	Placebo intake led to a decrease in severity and activity of gastritis in the antrum (inflammatory cell score after 3-wk and 16 wk consumption: 6.3 ± 0.7 and 6.4 ± 1.0, respectively). In the placebo group, mucus depletion scores remained at the same level during the whole duration of the study. *H. pylori* density decreased in 38% of subjects after 3 wk and 50% after 16 wk
Horie *et al*[40], 2004, Japan, South Korea, Egypt	Human	1-	-	42 subjects with *H. pylori* infection	CCT	Two groups- A- test group (yogurt containing 1, 5 g of egg yolk IgY-urease 3 times daily) (n = 22), B- control group (IgY-urease free yogurt) (n = 20)	Effect of IgY-Urease drinking yogurt on C-UBT values	TG showed a reduction in UBT values from 51.18 ± 3.40 at wk 0 to 33.70 ± 3.50 and 31.03 ± 3.54 at 2 and 4 wk resp. Suppression of *H. pylori* infection in humans could be achieved by consumption of drinking yogurt fortified with IgY-urease	CG showed some decrease in UBT values from 51.40 ± 4.48 to 44.38 ± 5.17 and 43.53 ± 5.48 at 0, 2 and 4 wk, resp. There was no significant difference obtained at week 0 and weeks 2 or 4
Sakamoto *et al*[41], 2001, Japan	Human	2-		31 subjects infected with *H. pylori* infection	CT	The study was conducted in two parts. 1st part = 90 g of yogurt (0-9 wk). 2nd part = 90 g yogurt containing LG21 (9-18 wk)	Efficacy of *Lactobacillus gasseri* OLL2716 (LG21) as a probiotic for *Helicobacter pylori*	The [13C] urea breath test and assays of serum pepsinogens revealed a significant improvement following LG21 treatment. LG21 was thus determined to be effective in both suppressing *H. pylori* and reducing gastric mucosal inflammation	There was no significant difference in C-UBT levels at 0 (26.2 ± 15.1) and 9 (26.6 ± 13.7) wk

[1]Levels of evidence: 1++ High quality meta-analysis, systematic reviews of RCTs, or RCTs with a very low risk of bias; 1+ Well conducted meta-analysis, systematic reviews of RCTs or RCTs with a low risk of bias; 1- Meta-analysis, systematic reviews or RCTs or RCTs with a high risk of bias; 2++ High quality systematic reviews of case-control or cohort studies or high quality case-control or cohort studies with a very low risk of confounding, bias, or chance and a high probability that the relationship is causal; 2+ Well conducted case control or cohort studoes with a low risk of confounding, bias, or chance and a significant risk that the relationship is not causal; 3 Non-analytic studies, *e.g.*, case reports, case series; 4 Expert opinion. [2]Quality rating for individual studies: ++ Applies if all or most criteria from the checklist are fulfilled; where criteria are not fulfilled the conclusions of the study or review are thought very unlikely to alter; + Applies if some of the criteria from the checklist are fulfilled; where criteria are not fulfilled or are not adequately described, the conclusions of the study or review are thought unlikely to alter; - Applies if few or no criteria from the checklist are fulfilled; where criteria are not fulfilled or are not adequately described, the conclusions of the study or review are thought likely or very likely to alter. *H. pylori*: *Helicobacter pylori;* RCT: Randomised controlled trial; CCT: Controlled clinical trial; CT: Clinical trial; C-UBT: ^{13}C-urea breath test.

preparations in Helicobacter eradication can, at best, be classified as "equivocal" (3 trials with weak methodology and equivocal results). This apprehension is further substantiated by a meta-analytic sub-analysis presented in an earlier report[42]. In this sub-analysis the beneficial effect of these preparations was minimal and it failed on exclusion sensitivity analysis (exclusion of one study majorly altered results) in consonance with the hypothesized argument.

In the context of studies comparing FMPP with fermented milk, several results are noteworthy. Of the three trials reporting control group data, two (one RCT and one CCT; Evidence grade 1-)[39,40] documented an improvement in gastritis or C-UBT values in the control group which is consistent with the argument presented earlier. In the third pre- and post-intervention trial (clinical trial, evidence grade 2[41]) no significant differences were observed during the period that yogurt was administered alone. Hence, although there are some discrepant results the preponderance of the available evidence appears con-

Table 4 Whey protein components and its basic properties

Whey components	Concentration (g/L)	% of Whey Protein	Molecular weight (kDa)	Number of amino acids residues	Biological properties	Recommendation grade against Helicobacter[1]
β-Lactoglobulin	1.3	50%-55%	18277	162	Source of essential and branched chain amino acids	-
α-Lactalbumin	1.2	20%-25%	14175	123	Primary protein found in human breast milk Source of essential and branched chain amino acids	D
Immunoglobulins (A, B and C)	0.7	10%-15%	25000 (light chain) + 50000-70000 (heavy chain)	-	Primary protein found in colostrum Immune modulating benefits	D
Lactoferrin	0.1	1%-2%	80000	700	Antioxidant Antibacterial, antiviral, and antifungal Promotes growth of beneficial bacteria Naturally occurs in breast milk, tears, saliva, bile, blood, and mucus	A
Lactoperoxidase	0.03	0.50%	70000	612	Inhibits growth of bacteria	-
Bovine Serum Albumin	0.4	5%-10%	66267	582	Source of essential amino acids Large protein	-
Glycomacropeptide	1.2	10%-15%	6700	64	Source of branched chain amino acids Lacks the aromatic amino acids phenylalanine, tryptophan and tyrosine	D

[1]Grades of recommendations: A: At least one meta-analysis, systematic review, or RCT rated as 1++ and directly applicable to the target population or A systematic review of RCTs or a body of evidence consisting principally of studies rated as 1++ directly applicable to the target population and demonstrating overall consistency of results; B: A body of evidence including studies rated as 2++ directly applicable to the target population and demonstrating overall consistency of results or Extrapolated evidence from studies rated as 1++ or 1+; C: A body of evidence including studies rated as 2+ directly applicable to the target population and demonstrating overall consistency of results or Extrapolated evidence from studies rated as 2++; D: Evidence level 3 or 4 or Extrapolated evidence from studies rated as 2+.

sistent with the hypothesis that whey milk proteins may partly or completely explain the anti-Helicobacter properties of fermented milk based probiotic preparations.

Overall, the recommendation for fermented milk may be classified as Recommendation Grade-A. The magnitude of the benefit achieved by FMPPs is small (10%) but holds across a variety of preparations. FMPPs also carry the potential inherent advantage of better patient acceptability. Thus, they could offer a viable alternative for complementing traditional regimens. Further research is necessary to identify the active substrate/s and to define the exact product to be used, the optimal clinical setting (prevention/treatment, first line therapy/recurrence, symptomatic/asymptomatic, gastritis/ulcer diseases, treatment failure *etc.*) and potential benefits in the setting of high antibiotic resistance.

WHEY PROTEINS

Whey proteins are globular water soluble molecules constituting 20% of the milk protein system. The whey protein profile, including general chemical, physicochemical and biological properties is depicted in Table 4. β-LG comprises the maximum percentage of whey protein but it has not been documented to possess any anti-bacterial properties. Other proteins have promising antibacterial

attributes and hence have been studied in *in vitro*, *in vivo* and in human trials. With specific reference to *H. pylori* infection and associated conditions lactoferrin, α-LA, glycomacropeptide and immunoglobulins appear to be potentially relevant and warrant further discussion.

Bovine lactoferrin

Bovine lactoferrin, an iron-binding glycoprotein, is a non-enzymatic antioxidant found in the whey fraction of fermented milk as well as in colostrum. The possibility that bLF may help to improve the *H. pylori* eradication rate was first conceived in 1997 when, in an *in vitro* study by Yamazaki *et al*[43], bLf was found to be bactericidal to *H. pylori* in Brucella broth. Later *in vitro* studies have confirmed the same and yielded evidence of the possible mechanism of bactericidal action of bLf relating it to the high iron-binding affinity and prevention of iron utilization by *H. pylori*[44,45]. An additional mechanism based on the interaction of bLf with the bacterial surface is also suggested in the context of bactericidal effect on *S. mutans* and *V. cholerae*[46]. It has been observed that bLf can bind to the outer membrane of Gram-negative bacteria and trigger the release of lipopolysaccharides, and kill the bacteria through osmotic damage[47,48]. Building on the available evidence Wada *et al*[49], in their study, examined the therapeutic effect of bLf on *H. pylori* infection using *in vitro* and *in vivo* experimental systems. In the experiment a significant inhibition of *H. pylori* binding to gastric epithelium was accomplished within 8 h after incubation. As a follow up experiment mice infected with *H. pylori* were given 10 mg of bLf orally every day and their stomachs were removed after 2 wk. 40.0% of all *H. pylori* attached themselves to the epithelium in the stomach of the untreated mice, whereas only 19.9% of the *H. pylori* did in the bLf-treated mice. However, in a similar experiment by Huynh *et al*[50], bLF, desferrioxamine and human recombinant lactoferrin had positive *in vitro* effects but all three failed to reduce *H. pylori* load in mice.

The above experimental evidence led to several human clinical trials. These are summarized in Table 5[43,51-57]. As presented, 5 (of 7 available) positive clinical trials and a meta-analysis appear to establish the beneficial effect of bLf (4%-17% as per meta-analysis) on *H. pylori* eradication fairly well[58]. The positive response was variously explained by the authors: (1) synergistic action of the antibiotics with bLf against *H. pylori*; (2) Inhibition of *Helicobacter* growth in an acidic pH by bLf; (3) Ability of bLf to bind to iron inhibiting growth of *H. pylori*; and (4) decrease in incidence of side effects and non-compliance. Two studies by Zullo *et al*[56] and Imoto *et al*[57] did not show any significant difference on addition of lactoferrin to triple therapy. In the first study this could be explained by the lack of synergism between lactoferrin and amoxicillin[56]. Alternatively, the anti-bacterial effect of lactoferrin based on bacterial membrane damage of Gram negative bacteria could be marginalized when amoxicillin is administered. In the second study the authors using quadruple therapy (rabeprazole, clarithromycin, tinidazole and lactoferrin) showed a statistically insignificant improvement in the eradication rate (4% in ITT analysis and 7% in per-protocol analysis). The results of this trial are limited by marked geographical heterogeneity (multicentre trial) in eradication rates.

Although the available evidence suggests that bLf is beneficial (Recommendation Grade-A), the magnitude of the documented benefit is small. Given that it lacks the inherent advantage in patient acceptability (requires to be given as a drug), the concept that fermented milk potentially has a clinically significant benefit (other than suggesting that whey protein may be partly/completely responsible for the benefit with FMPP) remains unclear. Its role in various clinical settings and more so in the presence of high antibiotic resistance deserves further exploration.

α-LA

α-LA is a major milk protein comprising 20-25% of whey proteins and has strong calcium binding ability. α-LA is reported to be biologically active *in vivo* with well-demonstrated antiulcer activity in rats. Matsumoto *et al*[59], in an *in vivo* study using ethanol ulcer model rats, documented 82% reduction of ulcerative lesion index using 200 mg/kg bw of α-LA. Similar results were reported by Mezzaroba *et al*[60], with absolute alcohol and indomethacin ulcer model rats given commercial α-LA. This intervention resulted in 30%-70% reduction in the ulcerative lesion index in comparison with controls. The exact mechanism of the protective effect and its impact on *Helicobacter* is not well studied. However, as reported, whey protein concentrates have consistently shown anti-Helicobacter properties. The minimal evidence on the subject precludes any definitive comment on the potential of α-LA as an anti-Helicobacter agent. The paucity of literature on the subject presents wide scope for future research.

Glycomacropeptide

Glycomacropeptide (GMP), also referred to as caseinomacropeptide and caseinoglycopeptide, is formed when bovine κ-casein is hydrolysed into para-κ-casein, which remains with the curd, and GMP, which is removed with the whey. It constitutes 15%-20% of whey protein. GMP has also been found to have several immunomodulatory functions and antibacterial properties. Otani *et al*[61] demonstrated that GMP, which contains sialic acid, inhibits the activity of *Salmonella typhimurium* lipopolysaccharide, inhibiting bacterial and viral adhesion especially to epithelial cells and dental plaque[62,63]. Other relevant properties like suppression of gastric secretions in dogs have been reported by a study group[64].

A study done in Japan attempted to enhance the ability of glycopeptides to bind pathogenic bacteria *in vivo* by conjugating with the non-digestible saccharides. The results of this study suggest that GMP could be a promising agent for preventing intestinal infection using its ability to bind pathogenic bacteria[65]. In the context

Table 5 Studies comparing bovine lactoferrin with placebo or "standard therapy + bovine lactoferrin" with "standard therapy"

Ref.	Type of trial	Evidence grade[1]	Quality rating[2]	Subjects	Study design	Study groups	Outcome variable	Results and conclusion
Sachdeva *et al*[58], 2009, India	Metaanalysis	1+	++	5 trials; 682 subjects [bLF group (n = 316); control group (n = 366)]	Metaanalysis of human RCTs/CCTs	Trials had to be randomized or quasi-randomized and controlled, using bLF in the intervention group treating *Helicobacter*-infected patients. The only difference between the two groups had to be bLF	Eradication of *H. pylori*; adverse events of eradication therapy	The pooled odds ratio (5-studies) for eradication by intention to treat analysis was 2.22 (95%CI: 1.44-3.44; P = 0.0003) using the fixed effects model (FEM) and 2.24 (95%CI: 1.15-4.35; P = 0.0003) using the random effects model (REM) (Cochran's Q = 6.83; P = 0.145). The pooled risk difference was 0.11 (95%CI: 0.05 -0.16; P = 0.0001) by FEM (Cochran's Q = 6.67; P = 0.154) and 0.10 (95%CI: 0.04-0.17; P = 0.0023) by REM. There was no significant difference in incidence of adverse effects
Di Mario *et al*[51], 2003, Italy	Human	1+	+	150 consecutive *H. pylori*-positive patients suffering from dyspeptic symptoms, gastritis and peptic ulcer disease	RCT	Three groups – A-triple therapy (rabeprazole,clarithromycin,tinidazole) with lactoferrin for 7 d (n = 51), B-triple therapy for 7 d (n = 52), C- triple therapy for 10 d (n = 47)	Efficacy of standard triple therapy plus bovine lactoferrin in the eradication of *H. pylori*	Eradication rates (ITT) were A-92.2%, B-71.2%, C-70.2 %. Results suggest that lactoferrin tested in the present study was effective in curing *H. pylori* and could be a new agent to assist the antimicrobials in the eradication of the bacterium
Di Mario *et al*[52], 2006, Italy	Human	1+	+	402 consecutive *H. pylori*-positive patients suffering from dyspeptic symptoms, gastritis and peptic ulcer disease	RCT	Three groups – A- triple therapy (esomeprazole, clarithromycin ,tinidazole) for 7 d (n = 136), B-lactoferrin followed by triple therapy for 7 d (n = 132), C- triple therapy with lactoferrin (n = 134)	Efficacy of bovine lactoferrin in the treatment of *H. pylori* infection	Eradication rate (ITT)- A- 77%, B-73%, C = 90%. Incidence of side effects was A- 9.5%, B- 9%, C- 8.2% Results demonstrate that bovine lactoferrin is an effective adjuvant to triple therapy for eradication of *H. pylori* Infection
Okuda *et al*[53], 2005, Japan	Human	1-	+	59 *H. pylori* infected healthy volunteers or children who were enrolled in a previous epidemiological study	CCT	Two groups- bLF (n = 31), placebo (n = 28)	Efficacy of a single administration of bLF. Improvement of *H. pylori* infection, adverse effects	Positive response (> 50% decrease in C-UBT values) was observed in 10 of 31 bLF-treated subjects and 1 of 28 control subjects, indicating that the rate of positive response in the bLF group was significantly higher than that in the control group
Tursi *et al*[54], 2007, Italy	Human	1-	+	70 consecutive patients with persistent *H. pylori* infection after failure of a first standard treatment	CCT	Two groups- A-quadruple therapy (ranitidine bismuth citrate plus triple therapy-esomeprazole ,amoxicillin, tinidazole) (n = 35), B- quadruple therapy plus lactoferrin (n = 35)	Efficacy and tolerability of bLF supplementation to this quadruple therapy in re-treating *H. pylori* infection	Eradication rate- A-88.57%, B-94.28%. Side effects- A-29.41%, B-17.64%. bLF supplementation was found effective in reducing side-effect incidence. It seems capable of achieving a slight (NS statistically) improvement in eradicating *H. pylori*

Zullo *et al*[55], 2005, Italy	Human	1+	++	133 consecutive patients with non-ulcer dyspepsia and *H. pylori* infection	RCT	Two groups- A- triple therapy for 7 d (n = 68), B- quadruple therapy (triple therapy plus lactoferrin) (n = 65)	Eradication rate of *H. pylori* infection, side effects and compliance	Eradication rate (ITT) A- 77.9%, B- 76.9%. Side effects- A –10.3%, B- 9.2%. Quadruple therapy with bLF did not significantly increase the *H. pylori* cure rate of standard 7-d clarithromycin-amoxycillin based triple therapy in non-ulcer dyspepsia patients
Zullo *et al*[56], 2007, Italy	Human	1+	+	144 consecutive dyspeptic patients	RCT	Two groups – A- triple therapy (rabeprazole, levofoxacin, amoxycillin) (n = 72), B- quadruple therapy (rabeprazole, clarithromycin, tinidazole plus bovine lactoferrin) (n = 72)	Eradication rate of *H. pylori* infection, side effects and compliance	Eradication rate (ITT) A- 68.1%, B-72.2%. *H. pylori* eradication rate following both quadruple therapy with lactoferrin and a low-dose PPI, triple therapy with levofloxacin is disappointingly low
Imoto *et al*[57], 2004	Human	FTNA	FTNA	25 *H. pylori* positive healthy volunteers	CCT	Two groups- A- bLf mixed with a commercial yogurt (n = 16) B- yogurt (n = 9)	Effect of bLf against *H. pylori*	The C-UBT values at week 8 were significantly lower than those at week 0 in the bLf group (P < 0.01), whereas no difference was observed in the control group

[1]Levels of evidence: 1++ High quality meta-analysis, systematic reviews of RCTs, or RCTs with a very low risk of bias; 1+ Well conducted meta-analysis, systematic reviews of RCTs or RCTs with a low risk of bias; 1- Meta-analysis, systematic reviews or RCTs or RCTs with a high risk of bias; 2++ High quality systematic reviews of case-control or cohort studies *or* High quality case-control or cohort studies with a very low risk of confounding, bias, or chance and a high probability that the relationship is causal; 2+ well conducted case control or cohort studoes with a low risk of confounding, bias, or chance and a significant risk that the relationship is not causal; 3 Non-analytic studies, eg case reports, case series; 4 Expert opinion. [2]Quality rating for individual studies: ++ Applies if all or most criteria from the checklist are fulfilled; where criteria are not fulfilled the conclusions of the study or review are thought very unlikely to alter; + Applies if some of the criteria from the checklist are fulfilled; where criteria are not fulfilled or are not adequately described, the conclusions of the study or review are thought unlikely to alter; - Applies if few or no criteria from the checklist are fulfilled; where criteria are not fulfilled or are not adequately described, the conclusions of the study or review are thought likely or very likely to alter. *H. pylori*: *Helicobacter pylori*; RCT: Randomised controlled trial; CCT: Controlled clinical trial; CT: Clinical trial; C-UBT: ^{13}C-urea breath test; FTNA: Full text not available; NS: Not significant.

of *Helicobacter* infection several authors have expressed the view that GMP has gastroprotective properties[66] but there is no direct evidence supporting its role in its eradication. Currently, in the absence of direct evidence the potential benefit of GMP in the treatment of *H. pylori* infection remains speculative.

Immunoglobulins

Immunoglobulins constitute a complex group, the elements of which are produced by B-lymphocytes. They make a significant contribution to the whey protein content (10-15%). Some of them attach to surfaces, where they behave as receptors, whereas others function as antibodies, which are released in the blood and lymph. Early *et al*[67], in an *in vitro* study, demonstrated that whey protein concentrates produced using milk from *H. pylori* immunized cows contain antibodies that are active at the pH of the stomach, and bactericidal against *H. pylori in vitro*. Oona *et al*[68], in their study on 20 children suffering from recurrent abdominal pain and with proven *H. pylori* infection, showed alleviation of gastritis and/or a decrease in the degree of colonization of the antrum mucosa in 9/14 children, and of the corpus mucosa in 7/15 children using immune colostrum of cows immunized (whole-cell vaccine prepared with *H. pylori* strain NCTC 11637) before calving. It is clear that evidence on the *in vivo* effects of the immunoglobulin in prevention or treatment of *H. pylori* infections in humans is only suggestive and deserves further work.

CONCLUSION

In conclusion, FMPP and bovine lactoferrin appear to be beneficial in *Helicobacter* eradication (Evidence Grade-A or -B in various settings with level 1++ studies available). Evidence for α-lactabumin and whey protein concentrates enriched in immunoglobulins is "suggestive of benefit". However the studies are small and/or based on animals (level 3 or 4 studies only; no grading possible). Literature on glycomacropeptide is very preliminary precluding relevant inferences. No studies directly comparing the efficacy of individual components amongst themselves or to FMPP were available. Overall, the magnitude of the potential benefit documented so far for the group is small and the precise clinical settings are poorly defined. This restricts more widespread use of this group as a complementary therapy in a nutraceutical setting hinging on better patient acceptability/compliance. Further work is necessary to identify the optimal substrate, fermentation process, dose of administration and the ideal clinical setting (prevention/treatment, first line therapy/recurrence, symptomatic/asymptomatic, gastri-

tis/ulcer diseases *etc.*). The potential of this group in high antibiotic resistance or treatment failure settings presents interesting possibilities and deserves further exploration.

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MINIREVIEWS

Solitary rectal ulcer syndrome: Clinical features, pathophysiology, diagnosis and treatment strategies

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Abstract

Solitary rectal ulcer syndrome (SRUS) is an uncommon benign disease, characterized by a combination of symptoms, clinical findings and histological abnormalities. Ulcers are only found in 40% of the patients; 20% of the patients have a solitary ulcer, and the rest of the lesions vary in shape and size, from hyperemic mucosa to broad-based polypoid. Men and women are affected equally, with a small predominance in women. SRUS has also been described in children and in the geriatric population. Clinical features include rectal bleeding, copious mucus discharge, prolonged excessive straining, perineal and abdominal pain, feeling of incomplete defecation, constipation, and rarely, rectal prolapse. This disease has well-described histopathological features such as obliteration of the lamina propria by fibrosis and smooth muscle fibers extending from a thickened muscularis mucosa to the lumen. Diffuse collage deposition in the lamina propria and abnormal smooth muscle fiber extensions are sensitive markers for differentiating SRUS from other conditions. However, the etiology remains obscure, and the condition is frequently associated with pelvic floor disorders. SRUS is difficult to treat, and various treatment strategies have been advocated, ranging from conservative management to a variety of surgical procedures. The aim of the present review is to summarize the clinical features, pathophysiology, diagnostic methods and treatment strategies associated with SRUS.

Key words: Solitary rectal ulcer syndrome; Pathophysiology; Diagnosis; Treatment; Clinical characteristics; Treatment

Core tip: We summarize the clinical features, pathophysiology, and diagnostic methods associated with solitary rectal ulcer syndrome (SRUS). Several therapies such as topical medication, behavior modification supplemented by fiber and biofeedback, and surgery are also discussed. The review might be conducive to understanding the nature of SRUS more systematically.

Zhu QC, Shen RR, Qin HL, Wang Y. Solitary rectal ulcer syndrome: Clinical features, pathophysiology, diagnosis and treatment strategies. *World J Gastroenterol* 2014; 20(3): 738-744 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i3/738.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i3.738

INTRODUCTION

Solitary rectal ulcer syndrome (SRUS) is a rare benign disorder characterized by a combination of symptoms, endoscopic findings, and histological abnormalities[1]. It was first described by Cruveihier[2] in 1829, when he reported four unusual cases of rectal ulcers. The term "solitary

ulcers of the rectum" was used by Lloyd-Davis in the late 1930s and in 1969 the disease became widely recognized after a review of 68 cases by Madigan *et al*[3], and few years later, a more comprehensive pathogenic concept of the disease was reported by Rutter *et al*[4]. SRUS is an infrequent and underdiagnosed disorder, with an estimated annual prevalence of one in 100000 persons. It is a disorder of young adults, occurring most commonly in the third decade in men and in the fourth decade in women. Men and women are affected equally, with a small predominance in women[5]. However, it has been described in children and in the geriatric population[6]. Solitary rectal ulcer is a misnomer because ulcers are found in 40% of patients, while 20% of patients have a solitary ulcer, and the rest of the lesions differ in shape and size, including hyperemic mucosa to broad-based polypoid lesions[7]. There is even a suggestion that the disease process also may involve the sigmoid colon[8].

In addition, the etiology is not known but may involve a number of mechanisms. For example, ischemic injury from pressure of impacted stools and local trauma due to repeated self-digitation may be contributing factors[9]. Furthermore, opinion differs regarding the best treatment for this troublesome condition, varying from conservative management and enema preparations to more invasive surgical procedures such as rectopexy[10]. In this mini-review, several aspects of this syndrome are evaluated, and detailed information about the disease will help guide future prevention and treatment strategies.

CLINICAL FEATURES AND PATHOPHYSIOLOGY

SRUS is a chronic, benign, underdiagnosed disorder characterized by single or multiple ulcerations of the rectal mucosa, with the passage of blood and mucus, associated with straining or abnormal defecation[11]. The average time from the onset of symptoms to diagnosis is 5 years, ranging from 3 mo to 30 years in adults, which is longer than in pediatric patients (1.2-5.5 years)[12]. Clinical features include rectal bleeding, copious mucus discharge, prolonged excessive straining, perineal and abdominal pain, feeling of incomplete defecation, constipation, and rarely, rectal prolapse[13,14]. The amount of blood varies from a little fresh blood to severe hemorrhage that requires blood transfusion[15]. Some children present with apparent diarrhea (because of prolonged visits to the bathroom), and associated bleeding, abdominal pain, and tenesmus suggest to clinicians the presence of inflammatory bowel disease[16]. However, it is unusual that a child may present with recurrent rectal bleeding and anemia requiring blood transfusion[6]. Although the passage of blood during defecation is the hallmark, up to 26% of patients can be asymptomatic, discovered incidentally when investigating other diseases[7].

The underlying etiology and pathogenesis are not fully understood but multiple factors may be involved. The most accepted theories are related to direct trauma or local ischemia as causes. It has been suggested that descent of the perineum and abnormal contraction of the puborectalis muscle during straining on defecation or defecation in the squatting position result in trauma and compression of the anterior rectal wall on the upper anal canal, and internal intussusceptions or prolapsed rectum[6,17]. Mucosal prolapse, overt or occult, is the most common underlying pathogenetic mechanism in SRUS. This may lead to venous congestion, poor blood flow, and edema in the mucosal lining of the rectum and ischemic changes with resultant ulceration. The cause of ischemia may also be related to fibroblasts replacing blood vessels, and pressure by the anal sphincter. Moreover, rectal mucosal blood flow has been found to be reduced in SRUS to a level similar to that seen in normal transit constipation, suggesting similar impaired autonomic cholinergic gut-nerve activity[18]. Self-digitation maneuver to reduce rectal prolapse or to evacuate an impacted stool may also cause direct trauma of the mucosa and ulceration[19]. Although this hypothesis seems plausible, it remains unproven because rectal mucosal intussusception is common even in healthy subjects, but rectal prolapse and SRUS are rare[20]. In addition, not all patients with rectal prolapse have SRUS and vice versa[21]. Furthermore, ulcers usually occur in the mid rectum, which can not be reached by digital examinations[22]. Hence, it has been suggested that rectal prolapse and SRUS are two disparate conditions. In children, secondary to chronic mechanical and ischemic trauma, inflammation by hard stools, and intussusceptions of the rectal mucosa, some histological features of SRUS can be seen, such as fibromuscular obliteration of the lamina propria and disorientation of muscle fibers[23].

Anorectal physiology studies have shown that 25%-82% of patients with SRUS may have dyssynergia with paradoxical anal contraction[24]. Studies have confirmed that uncoordinated defecation with excessive straining over time play a key role in SRUS[19]. Morio and his colleagues found that SRUS patients compared with three control groups (dyssynergic defecation alone, rectal prolapse with or without mucosal changes) had more frequent increase in anal pressure and paradoxical puborectalis contraction during strain[25]. In addition, a case-control study showed that up to 82% of subjects exhibited dyssynergia along with prolonged balloon expulsion time[19]. Also, SRUS patients exhibited rectal hypersensitivity, raising the hypothesis that hypersensitivity may lead to a persistent desire to defecate and/or feeling of incomplete evacuation and excessive straining.

DIAGNOSIS

The cause of SRUS is unknown. The clinical presentation varies, therefore, early diagnosis requires a high index of suspicion from both the surgeon and the pathologist[7,20], especially because the term "solitary rectal ulcer" is a misnomer and only a quarter of the adults with SRUS have a true rectal ulcer, and the lesion is not necessarily solitary

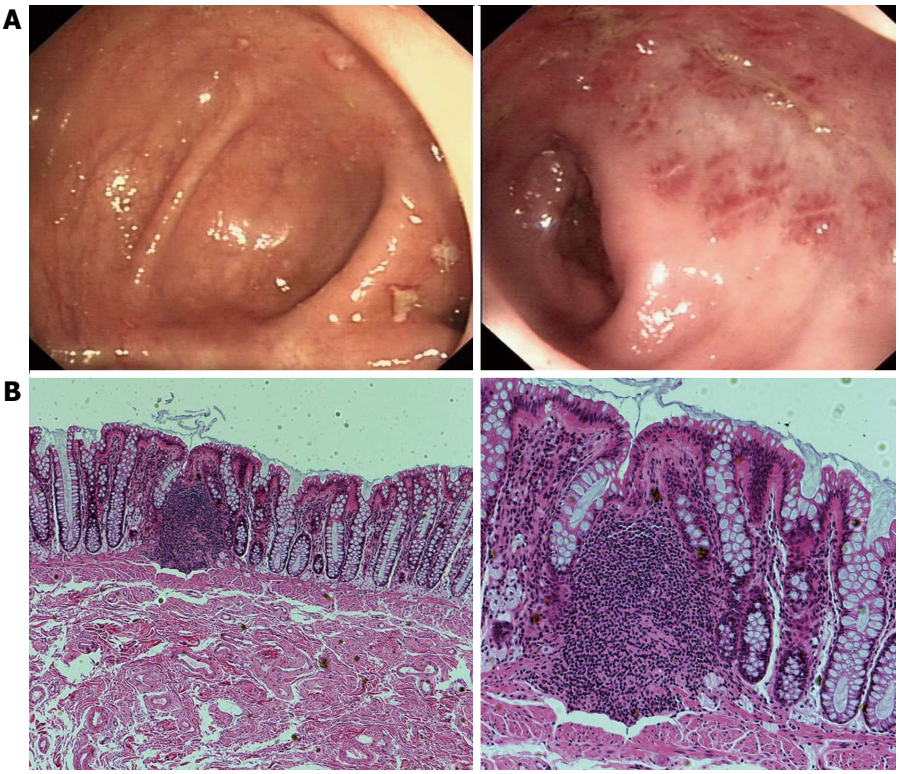

Figure 1 Endoscopic imaging and corresponding histological findings in solitary rectal ulcer syndrome patients. A: Colonoscopy revealed localized yellowish slough, rectal edema, erythema, and superficial ulcerations; B: Histology (hematoxylin and eosin) shows smooth muscle hyperplasia in the lamina propria between colonic glands, and surface ulceration with associated chronic inflammatory infiltrates. Magnification: × 40 (left), × 100 (right).

or ulcerated[26]. Diagnosis of SRUS is based on clinical features, findings on proctosigmoidoscopy and histological examination, imaging investigations including defecating proctography, dynamic magnetic resonance imaging, and anorectal functional studies including manometry and electromyography[27]. A complete and thorough history is most important in the initial diagnosis of SRUS. Differential diagnosis includes Crohn's disease, ulcerative colitis, ischemic colitis, and malignancy. Obstructive symptoms in children may be interpreted by parents as constipation. In a quarter of patients, a delay in diagnosis or misdiagnosis of SRUS might occur because of inadequate rectal biopsy and failure to recognize the histopathological features of the disease[27]. Concomitant hematochezia may be misinterpreted as originating from an anal fissure caused by constipation, or as other causes of rectal bleeding such as a juvenile polyp[11,28].

Colonoscopy and biopsy of normal and abnormal-looking rectal and colonic mucosa should be performed. It has been reported that the ulcer is usually located on the anterior wall of the rectum and the distance of the ulcer from the anal margin varies from 3 to 10 cm[3]. Ulcers may range from 0.5 to 4 cm in diameter but are usually 1-1.5 cm[29]. The appearance of SRUS on endoscopy may vary from preulcer hyperemic changes of rectal mucosa to established ulcers covered by a white, grey or yellowish slough[3,29] (Figure 1A). The ulceration is shallow and the adjacent mucous membrane may appear nodular, lumpy or granular[30]. Twenty-five percent of SRUSs may appear as a polypoid lesion; 18% may appear as patchy mucosal erythema; and 30% as multiple lesions. As a result of the wide endoscopic spectrum of SRUS and the fact that the condition may go unrecognized or, more commonly, misdiagnosed, it is crucial to collect biopsy specimens from the involved area to confirm the diagnosis and to exclude other diagnoses, including cancer[5]. Defecography is a useful method for determining the presence of intussusception or internal or external mucosal prolapse and can demonstrate a hidden prolapse, as well as a non-relaxing puborectalis muscle and incomplete or delayed rectal emptying[31]. However, because of the wide availability of endoscopy and biopsy, defecography usually is reserved for the investigation of the underlying pathophysiology and possibly for preoperative assessment[32]. Barium enema shows granularity of the mucosa, polypoid lesion, rectal stricture and ulceration, and thickened rectal folds; all of which are nonspecific findings[33]. It has been recommended that defecography and anorectal manometry should be performed in all children with SRUS to define the primary pathophysiological abnormality and to select the most appropriate treatment protocol[34]. Anorectal manometry and electromyography provide useful information about anorectal inhibitory reflex, pressure profiles, defecation dynamics, and rectal compliance and sensory thresholds. On awake anorectal manometry, 42%-55% of children with chronic constipation show dyssynergia and abnormal contraction of voluntary muscles of the pelvic floor and external anal sphincters

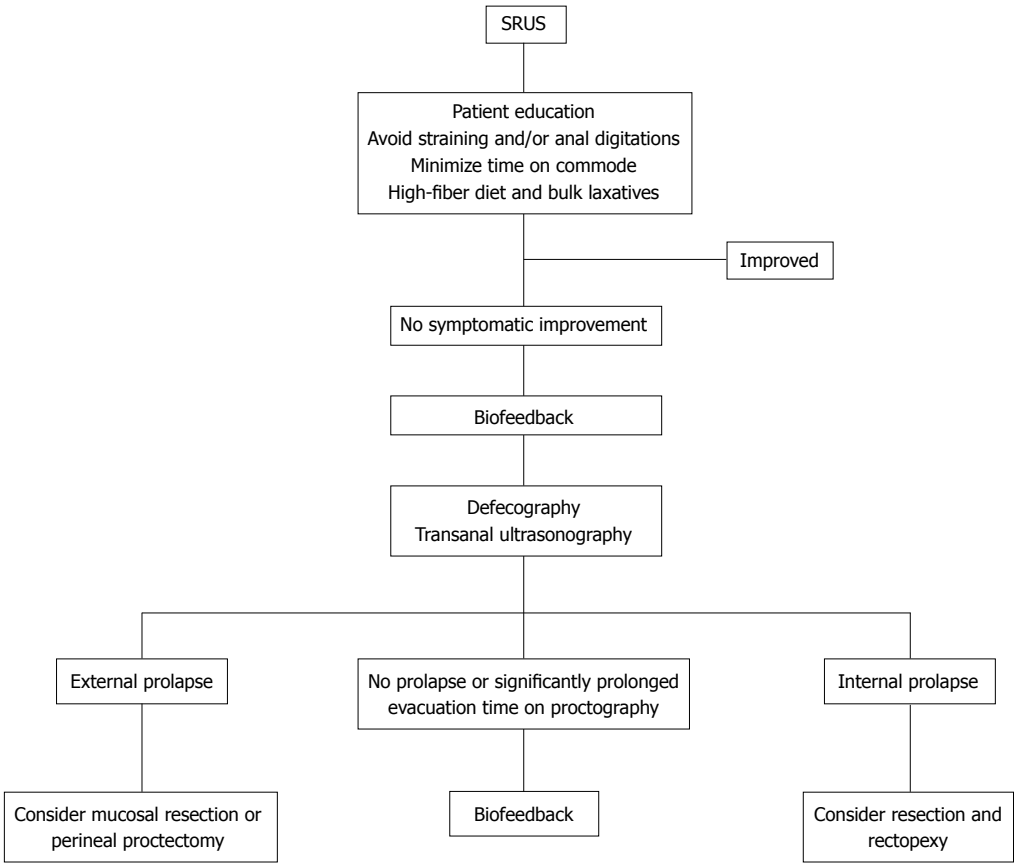

Figure 2 Suggested algorithm for treatment strategies in patients with solitary rectal ulcer syndrome. SRUS: Solitary rectal ulcer syndrome.

(EASs) during an attempt to expel a rectal balloon[35]. In adults, excessive straining and uncoordinated defecation, caused by dyssynergia of pelvic floor muscles, are attributed to development of SRUS[36]. These all suggest a relationship between dyssynergia of the pelvic floor and the EAS muscles, constipation, rectal prolapse, and SRUS. Recent studies have shown the usefulness of anorectal ultrasound in assessing internal anal sphincter thickness, which is increased in patients with SRUS[37], and it has been suggested that sonographic evidence of a thick internal anal sphincter is highly predictive of high-grade rectal prolapse and intussusceptions[38]. Routine laboratory tests including red and white blood cell counts, platelet count, hemoglobin, liver function tests, coagulation tests, C-reactive protein, and erythrocyte sedimentation rate are usually normal. Features of microcytic anemia with low values of hemoglobin, hematocrit and mean corpuscular volume may, however, be seen in a child with a history of recurrent bleeding per rectum[35].

Key histological features include fibromuscular obliteration of the lamina propria, hypertrophied muscularis mucosa with extension of muscle fibers upwards between the crypts, and glandular crypt abnormalities[39] (Figure 1B). Other minor microscopic changes, including surface erosion (which is covered by mucus, pus and detached epithelial cells, and may show reactive hyperplasia with distortion of the crypt architecture), mild inflammation, distorted crypts, and reactive epithelial atypia, may lead to erroneous diagnoses such as inflammatory bowel disease (which may show chronic and acute inflammatory cells in lamina propria, cryptitis, crypt abscesses and granuloma formation, with distortion of epithelial and glandular structures) and cancer[40]. Diffuse collagen deposition in the lamina propria and abnormal smooth muscle fiber extensions are sensitive markers for differentiating SRUS from other conditions[41].

TREATMENT

Several treatment options have been used for SRUS, ranging from conservative treatment (*i.e.*, diet and bulking agents), medical therapy, biofeedback and surgery (Figure 2). The choice of treatment depends upon the severity of symptoms and whether there is a rectal prolapse.

Patient education and behavioral modification are the first steps in the treatment of SRUS[9]. In particular, asymptomatic patients may not require any treatment other than behavioral modifications. Other suggestions for the treatment include reassurance of the patient that the lesion is benign, encouragement of a high-fiber diet, avoidance of straining, regulation of toilet habits, and attempt to discuss any psychosocial factors[42]. The use of a high-fiber diet, in combination with stool softeners and bulking laxatives, and avoidance of straining have had varying responses[43]. These dietary and behavioral modifications are especially effective in patients with mild to moder-

ate symptoms and with absence of significant mucosal prolapse. However, it would appear that conservative approaches are less useful when SRUS is associated with an advanced grade of rectal intussusception, extensive inflammation, established fibrosis and/or reducible external prolapse[44]. Therefore, in patients whose symptoms are resistant to those conservative measures, a more organized form of behavioral therapy such as biofeedback appears promising. It has been suggested that, in selected patients, biofeedback improves symptoms by altering efferent autonomic pathways to the gut[45]. Biofeedback includes reducing excessive straining with defecation by correcting abnormal pelvic-floor behavior and by attempting to stop the aid of laxatives, suppositories, and enemas[46]. In a case-control study, standard biofeedback therapy improved both anorectal function and bowel symptoms in most patients who exhibited dyssynergic defecation[19]. Furthermore, the improvement in symptoms and manometric findings was associated with significant healing in 54% of patients. In another study, Jarrett and his colleagues found that 12/16 (75%) patients with SRUS had subjective improvement after biofeedback, and this was associated with increased rectal mucosal blood flow, suggesting that improved extrinsic innervation to the gut could be responsible for such a successful response[36]. Some authors suggest that biofeedback helps in the short term, but is less effective in the long term, and further systematic studies in a large population are required[42].

Topical treatments, including sucralfate, salicylate, corticosteroids, sulfasalazine, mesalazine and topical fibrin sealant, have been reported to be effective with various responses and improvement of symptoms[47]. Sucralfate enema contains aluminum complex salts, which coat the rectal ulcer and form a barrier against irritants, allowing the ulcer to heal. Corticosteroids and sulfasalazine enemas may also help ulcer healing by reducing the inflammatory responses. However, these treatments are empirical and have been applied in uncontrolled studies, and their long-term benefits deserve further investigation[48,49].

Surgery remains an option for patients not responsive to conservative measures and biofeedback. Surgery is warranted in almost one-third of adults with associated rectal prolapse; in children this has only been described in case reports[10]. Surgical treatments include excision of the ulcer, treatment of internal or overt rectal prolapse, and defunctioning colostomy[47]. The indication for surgery is failure of conservative treatment to control severe symptoms, and the aim is to avoid formation of colostomy as a primary operation. Sclerotherapy injection into the submucosa or retrorectal space with 5% phenol, 30% hypertonic saline or 25% glucose and perianal cerclage is effective in treating rectal prolapse. A therapeutic role of botulinum toxin injection into the external anal sphincter for the treatment of SRUS, and constipation associated with dyssynergia of defecation dynamics has also been reported by Keshtgar *et al*[50]. The effect of botulinum toxin lasts approximately 3 mo, which may be more beneficial than biofeedback therapy. In addition, in children, laparoscopic rectopexy using a polypropylene mesh on each side of the rectum, fixed to sacral promontory with a nonabsorbable structure, has been used successfully to treat SRUS[10]. Furthermore, for full-thickness prolapse, mucosal resection (Delorme's procedure) or perineal proctectomy (Altemeier's procedure) has been advocated[51]. In a series of 66 adult patients with SRUS, rectopexy was done in 49, Delorme's operation in nine, restorative anterior resection in two, postanal repair and division of puborectalis in two, and primary colostomy in four[52]. Local excision of polypoid rectal ulcer and rectopexy for overt rectal prolapse, however, have a higher long-term cure rate[53]. Proctectomy may be required in patients with intractable rectal pain and bleeding, who have not responded to other surgical treatments[54]. Based on postoperative evacuation defecography studies, it has been shown that rectopexy alters rectal configuration and successfully treats rectal prolapse in SRUS, and that a prolonged preoperative evacuation time is predictive of poor symptomatic outcome[32]. When the above measures fail, mucosal-sleeve resection with coloanal pull-through or a diverting colostomy should be considered. The evidence regarding which approach is first-line for SRUS is unclear. However, open rectopexy and mucosal resection seem popular with a success rate of 42%-100%[55].

CONCLUSION

SRUS is a chronic, benign disorder in young adults, often related to straining or abnormal defecation. The pathogenesis of SRUS is not well understood, but may be multifactorial. Usually, patients present with straining, altered bowel habits, anorectal pain, incomplete passage of stools, and passage of mucus and blood. The diagnosis can be made clinically, endoscopically, and histologically. Symptoms may resolve spontaneously or may require treatment. A variety of therapies have been tried. Several therapies thought to be beneficial include topical medication, behavior modification supplemented by fiber and biofeedback, and surgery. Patient education and a conservative, stepwise individualized approach are important in the management of this syndrome.

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ORIGINAL ARTICLE

Transarterial chemoembolization in Barcelona Clinic Liver Cancer Stage 0/A hepatocellular carcinoma

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Abstract

AIM: To evaluate the clinical characteristics of patients with Barcelona Clinic Liver Cancer (BCLC) stage 0 and A hepatocellular carcinoma (HCC) after transarterial chemoembolization (TACE).

METHODS: Between January 2001 and September 2011, 129 patients with BCLC stage 0 and stage A HCC who underwent TACE were retrospectively enrolled. Patient characteristics, routine computed tomography and TACE findings, survival time and 1-, 5-, and 10-year survival rates, risk factors for mortality, and survival rates according to the number of risk factors were assessed.

RESULTS: The mean size of HCC tumors was 2.4 ± 1.1 cm, and the mean number of TACE procedures performed was 2.5 ± 2.1. The mean overall survival time and 1-, 5-, and 10-year survival rates were 80.6 ± 4.9 mo and 91%, 63% and 49%, respectively. In the Cox regression analysis, a Child-Pugh score > 5 (P = 0.005, OR = 3.86), presence of arterio-venous shunt (P = 0.032, OR = 4.41), amount of lipiodol used (> 7 mL; P = 0.013, OR = 3.51), and female gender (P = 0.008, OR = 3.47) were risk factors for mortality. The 1-, 5-, and 10-year survival rates according to the number of risk factors present were 96%, 87% and 87% (no risk factors), 89%, 65%, and 35% (1 risk factor), 96%, 48% and unavailable (2 risk factors), and 63%, 17%, and 0% (3 risk factors), respectively (P < 0.001).

CONCLUSION: TACE may be used as curative-intent therapy in patients with BCLC stage 0 and stage A HCC. The Child-Pugh score, arterio-venous shunt, amount of lipiodol used, and gender were related to mortality after TACE.

Key words: Carcinoma; Hepatocellular; Chemoembolization; Therapeutic; Survival; Stage; Efficacy

Core tip: In this study, transarterial chemoembolization

(TACE) was associated with a relatively good survival rate in patients with stage 0 and stage A Barcelona Clinic Liver Cancer (BCLC). The Child-Pugh score, presence of arterio-venous shunt, amount of lipiodol used during TACE, and female gender were correlated with mortality in patients with BCLC stage 0 and stage A hepatocellular carcinoma who underwent TACE. Patients with more than 2 risk factors should be treated by other curative-intent treatments after the first TACE.

Kim HC, Suk KT, Kim DJ, Yoon JH, Kim YS, Baik GH, Kim JB, Kim CH, Sung H, Choi JY, Han KH, Park SH. Transarterial chemoembolization in Barcelona Clinic Liver Cancer Stage 0/A hepatocellular carcinoma. *World J Gastroenterol* 2014; 20(3): 745-754 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i3/745.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i3.745

INTRODUCTION

Hepatocellular carcinoma (HCC) is a major health problem and is the sixth most common neoplasm in the world[1]. The incidence of HCC is increasing in Europe and the United States, and it is currently the leading cause of death among cirrhotic patients[2-4]. Eighty percent of patients with HCC have cirrhosis. The annual incidence of HCC in patients with cirrhosis is 3%-5%[2]. Hepatitis B virus infection is the main risk factor in Asia and Africa[5]. In Western countries, hepatitis C virus infection is the main risk factor[6,7]. The rising incidence of HCC has sparked widespread interest in research regarding the clinical management of HCC.

The Barcelona Clinic Liver Cancer (BCLC) staging system was constructed based on the results obtained in several randomized controlled and cohort studies[8-10]. The BCLC staging system has been validated by several groups in Europe and the United States[11-13], and the treatment of HCC has dramatically changed in the last few years[14]. Patients with BCLC stage 0 and stage A HCC are candidates for curative therapies such as resection, transplantation or radiofrequency ablation. The survival rate of patients with BCLC stage 0 and stage A HCC approaches 50%-70% at 5 years after curative therapy[15]. Five-year recurrence rates vary according to the therapeutic modality[16-18].

Transarterial chemoembolization (TACE) is the only intervention recommended by current HCC treatment guidelines for intermediate-stage patients[19]. In general, TACE has not been recommended as a first-line therapy for patients with BCLC stage 0 and stage A HCC, based on the results of a single retrospective study[20]. However, this study reported the results of transarterial embolization, not TACE, and little evidence comparing TACE with other curative therapies in patients with early stage HCC is available[21,22]. Thus, considerable controversy still remains regarding appropriate patient selection for TACE. In this 10-year retrospective study, we evaluated the clinical characteristics of patients with BCLC stage 0 and stage A HCC after TACE.

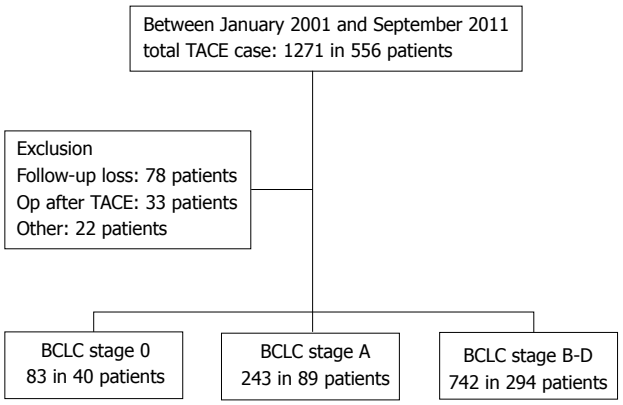


Figure 1 Study design. A total of 129 patients with Barcelona Clinic Liver Cancer (BCLC) stage 0 (n = 40) and stage A hepatocellular carcinoma (HCC) (n = 89) who underwent transarterial chemoembolization (TACE) were retrospectively enrolled. Op: Operation.

MATERIALS AND METHODS

Between January 2001 and September 2011, a total of 129 patients with BCLC stage 0 (n = 40) and stage A HCC (n = 89) who underwent TACE were retrospectively enrolled (Figure 1). All of these patients were either unable to or refused to undergo resection, transplantation, or ablative therapy. Contraindications for these modalities included an unacceptably high risk of surgery, unacceptably high risk of ablation due to the location of mass (close to the gallbladder, liver hilum, liver capsule, diaphragm or pericardium), financial constraints, or patient refusal. Thirty-three patients who were treated with surgery after TACE were excluded. Patient characteristics, routine pre- and post-treatment computed tomography (CT) findings, TACE findings, and 1, 5 and 10-year survival rates were reviewed and assessed. The study protocol conformed to the ethical guidelines established by the 1975 Declaration of Helsinki and received *a priori* approval by the participating hospitals' institutional review boards for human research.

Baseline evaluations were conducted including family and alcohol history, X-ray, electrocardiography, electrolyte panels, liver function tests, and viral markers. The diagnosis of HCC was established by α-fetoprotein (AFP) > 200 ng/mL, liver biopsy, and imaging tests, which included magnetic resonance image or contrast-enhanced CT scanning in the arterial and portal venous phases. Reviews of CT and TACE imaging were performed by a single radiologist (H.C.K.) who had over 15 years of experience with the TACE procedure. The clinical characteristics and medical reports were reviewed by 1 hepatologist (K.T.S.). The diagnosis of liver cirrhosis was established by liver biopsy and/or imaging tests such as ultrasound and/or contrast-enhanced CT in conjunction with laboratory data and by observing clinical complications of cirrhosis (presence of ascites, hepatic encephalopathy, and esopha-

geal varices). Kaplan-Meier survival analysis and Cox regression analysis were used to investigate risk factors for mortality. Survival rates, as classified by the number of risk factors, were also evaluated and compared.

TACE procedure

All patients gave informed consent prior to the procedure. All TACE procedures were performed using the Seldinger technique by a board-certified attending interventional radiologist who specialized in interventional oncology (H.C.K.)[23]. After arterial access was obtained via the common femoral artery, a 5-French catheter (RH, ANA MD Company, Seoul, South Korea) was introduced, and diagnostic angiography was performed of the celiac axis and superior mesenteric artery to assess arterial anatomy and to confirm patency of the portal vein. After the tumor feeder vessel was identified, a 2.9-French microcatheter (ASAHI Stride Microcatheter, Vascular Perspectives Ltd, Manchester, United Kingdom) was coaxially inserted through a 5-French catheter and advanced into the hepatic artery supplying the targeted tumor. Depending on the size, location, and blood supply, the tip of the catheter was advanced into the hepatic artery and the feeding branch.

After appropriate catheter placement, a chemotherapeutic emulsion of 2-10 mL of iodized oil (Lipiodol Ultra-Fluide, Andre Guerbet Laboratories, Anlney-Sous-Bois, France) and 10-50 mg of doxorubicin (Adriamycin, Ildong pharmaceutical CO. LTD, Seoul, South Korea) was injected. The doxorubicin-iodized oil emulsion was prepared by dissolving doxorubicin into a solution of nonionic water-soluble contrast medium and saline solution and mixed with lipiodol by shaking manually approximately 10 times. The dose of anticancer agent used for the TACE procedure was determined by the radiologist based on the size, number, and blood supply of the target tumors. The maximum dose of doxorubicin for a single TACE session was 50 mg. The injection was performed under fluoroscopy. If the slowing of antegrade blood flow was achieved, the chemotherapeutic infusion was discontinued, and subsequent embolization was performed using a gelatin sponge (Cutanplast, Mascia-Brunelli, Spa, Italy). In patients with decompensated liver function (classified as patients with Child-Pugh class C disease), if a large amount of intratumoral liver tissue was at risk for infarction due to reflux of emboli or segmental TACE, gelatin sponge embolization was not performed. TACE was terminated when the hepatic vein was visualized, the tumor vessels were completely filled with drug, and the tumor blush disappeared on subsequent angiographic imaging.

After the initial TACE treatment, patients underwent CT scans at 1, 3 and 6 mo after the procedure to evaluate the status of their tumors. In the case of an incomplete TACE, as evidenced by tumor recurrence or progression, a second TACE was performed after the follow-up CT scan.

Response after TACE

Imaging response was classified according to the Response Evaluation Criteria in Solid Tumors (RECIST), the European Association for the Study of the Liver (EASL), and the Modified RECIST (mRECIST) criteria[24,25]. According to RECIST, a complete response (CR) is defined as the disappearance of all target lesions. A partial response (PR) is defined as a 30% minimum decrease in the sum of the longest diameter of the target lesions, taking as reference the baseline sum of the longest diameter. Progressive disease (PD) is designated as a 20% minimum increase in the sum of the longest diameter of the target lesions, taking as reference either the smallest sum of the longest diameter recorded since the start of treatment or the appearance of one or more new lesions. Stable disease (SD) is indicated by insufficient tumor shrinkage to qualify for PR. According to the EASL guidelines, CR is defined as the absence of enhancing tumor areas, reflecting complete tissue necrosis. PR is defined as a > 50% decrease in the enhancing areas, reflecting partial tissue necrosis. PD is defined as a > 25% increase in the size of a single, measurable lesion or the appearance of new lesions, and SD is defined as a tumor response between PR and PD. The response categories, according to the criteria of mRECIST, are as follows: CR is defined as a disappearance of any intra-tumoral arterial enhancement in all target lesions; PR is defined as at least a 30% decrease in the sum of the diameters of viable target lesions; SD is defined as any cases that do not qualify for either CR or PD; and PD is defined as an increase of at least 20% in the sum of the diameters of viable target lesions.

Time to recurrence was defined as time from treatment to recurrence. Time to progression was defined as the time between treatment and radiological progression. The definitions of recurrence and progression were based on the mRECIST amendments. Patients alive and free of recurrence or progression at the end of follow-up were censored. Progression-free survival time was defined as the time between treatment and either radiological progression or death[24].

Statistical analysis

Quantitative data were expressed as the mean ± SD, unless otherwise stated. Numerical differences between the groups classified by categorical variables were assessed using the Pearson χ^2 test. To evaluate differences between the continuous variables among the groups, the independent t test was used. Survival was expressed as the mean (SE). Mean survival times were obtained using the Kaplan-Meier method and the Log-rank test. Cox regression analysis was used to investigate independent predictors of mortality. Age, gender and other plausible risk factors from the results of the univariate analysis ($P < 0.500$) were used in the Cox regression analysis. For the Cox regression analysis of lipiodol and doxorubicin, an area under the receiver operating characteristic was used to select the appropriate amount (lipiodol 7 cc and doxorubicin 27 cc). Subsequently, the Enter method was used to determine the OR and the risk factors for the Cox regression analysis. Risk factors are presented in

Table 1 Clinical characteristics of patients *n* (%)

Variables	BCLC stage 0 (*n* = 40)	BCLC stage A (*n* = 89)	*P* value
Age (yr)	59.5 ± 10.0	61.7 ± 11.0	0.208
Male	28 (73)	64 (67)	0.836
Etiology			
HBV	18 (45)	40 (45)	0.822
HCV	5 (13)	9 (10)	
Alcohol	10 (25)	21 (24)	
Others	7 (17)	19 (21)	
Child-Pugh score	5.4 ± 1.0	5.9 ± 0.9	0.976
Cirrhosis	38 (95)	73 (85)	0.146
Albumin (g/dL)	3.9 ± 0.7	3.9 ± 2.2	0.904
AST (IU/L)	67.7 ± 68.3	91.4 ± 217.2	0.503
ALT (IU/L)	43.2 ± 40.1	62.2 ± 133.1	0.378
TB (mg/dL)	1.2 ± 1.3	1.2 ± 0.9	0.971
GGT (IU/L)	98.9 ± 98.9	136.3 ± 244.9	0.398
N of TACE treatment	2.1 ± 1.5	2.7 ± 2.5	0.066
AFP (ng/mL)	75.6 ± 159.6	490.7 ± 1582.4	0.018
Greatest diameter of mass (cm)	1.4 ± 0.3	2.8 ± 1.0	< 0.001

BCLC: Barcelona Clinic Liver Cancer; HBV: Hepatitis B virus; HCV: Hepatitis C virus; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; TB: Total bilirubin; GGT: Gamma glutamyl transferase; AFP: α-fetoprotein.

Table 2 Analysis for the risk factors of mortality *n* (%)

Variables	Mortality (*n* = 41)	Survival (*n* = 88)	*P* value
Male	27 (66)	65 (74)	0.405
Age (yr)	60.9 ± 9.8	60.9 ± 11.2	0.956
Alcohol history	23 (56)	52 (59)	0.846
Smoking history	29 (71)	59 (67)	0.835
Presence of cirrhosis	37 (90)	77 (88)	0.772
BCLC class (A0/A1)	7/34	33/55	0.024
Number of nodule	36/2/3	79/8/1	0.129
Number of feeding vessel (1/2/≥ 3)	34/0/7	64/1/23	0.471
Child-Pugh score	6.1 ± 1.0	5.6 ± 0.9	0.013
Greatest diameter of nodule (cm)	2.5 ± 1.0	2.3 ± 1.1	0.383
Number of TACE treatment	3.3 ± 2.5	2.2 ± 2.0	0.010
Amount of doxorubicin used (mg)	29.4 ± 13.2	25.9 ± 12.4	0.156
Amount of lipiodol used (mL)	7.3 ± 3.7	5.9 ± 3.4	0.041
Presence of arterio-venous shunt	9 (20)	9 (10)	0.284
Use of gelatin sponge	21 (51)	35 (40)	0.239
Complete lipiodol uptake	31 (76)	63 (72)	0.492
Sub-segmental approach of catheter	32 (78)	66 (75)	0.624
Extrahepatic collateral supply	1 (2)	2 (2)	1.000
Visible hepatic vein during TACE	34 (83)	73 (83)	0.711

BCLC: Barcelona Clinic Liver Cancer; TACE: Transarterial chemoembolization.

terms of the OR and 95%CI. The times to recurrence and progression, recurrence-free survival, and progression-free survival were estimated using the Kaplan-Meier method. Data were analyzed using statistical software (SPSS, version 13.0, SPSS Inc., Chicago, IL, United States). A P value < 0.05 was considered significant for all tests.

RESULTS

Patient characteristics

A total of 129 patients were enrolled in this study (40 with BCLC stage 0 and 89 with BCLC stage A). The mean size of the HCC tumors was 2.4 ± 1.1 cm, and the mean number of TACE procedures performed was 2.5 ± 2.1. There were no significant differences among the continuous variables between the BCLC stage 0 and BCLC stage A groups (P > 0.050), with the exception of AFP (P = 0.018) and the greatest diameter of a nodule (P < 0.001). There were no significant differences in the distribution of etiology between the BCLC stage 0 and BCLC stage A groups (P = 0.822). There were 111 patients (38 in the BCLC stage 0 group and 73 in the BCLC stage A group) who showed evidence of cirrhosis in this study (Table 1).

Findings of CT and TACE

An analysis of CT and TACE imaging revealed that 115 patients had 1 nodule, 10 patients had 2 nodules and 4 patients had 3 nodules. Tumors were located in the left lobe of the liver in 18 patients, in the right lobe in 103 patients, and in both the left and right lobes in 8 patients. The greatest tumor diameter was 2.4 ± 1.1 cm. In 4 patients, the nodule was associated with a capsule in the CT scan. The amounts of doxorubicin and lipiodol used were 26.2 ± 12.5 mg and 6.1 ± 3.5 mL, respectively. Complete lipiodol uptake was observed in 101 patients, and a gelatin sponge was used in 56 patients. Ninety-four patients had 1 feeding vessel, 5 patients had 2 feeding vessels, and 30 patients had more than 3 feeding vessels. Eighteen patients had arterio-venous shunting, while 3 patients presented with an extra-hepatic collateral blood supply.

Risk factors of mortality

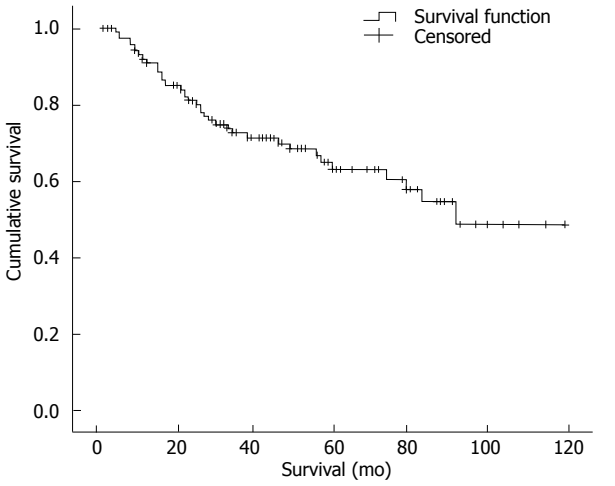
In the univariate analysis, the Child-Pugh score, BCLC class, number of TACE procedures, and amount of lipiodol used were correlated with mortality in patients with BCLC stage 0 and stage A HCC (P < 0.05). The other variables were not associated with mortality, as shown in Table 2.

In the Cox regression analysis, the Child-Pugh score > 5 (n = 67, P = 0.005, and OR = 3.86), the presence of an arterio-venous shunt (n = 18, P = 0.032, and OR = 4.41), the amount of lipiodol used (> 7 cc; n = 32, P = 0.013, and OR = 3.51) during TACE, and female gender (n = 92, P = 0.008, and OR = 3.47) were risk factors for mortality (Table 3). There was a positive correlation between nodule diameter and the amount of lipiodol used (r = 0.343, P < 0.001).

Survival of patients

The mean overall survival time and 1, 5, and 10-year survival rates were 80.6 ± 4.9 mo and 91%, 63% and 49%, respectively (Figure 2). Of the risk factors studied, only a Child-Pugh score of 5 was a statistically significant predictor of survival (P = 0.002). Other risk factors were not associated with a survival difference between groups (P > 0.050; Figure 3).

The mean survival times of patients with 0, 1, 2 and



Mean survival (SE)	1-yr	5-yr	10-yr
80.6 (4.9) mo	91%	63%	49%

Figure 2 Cumulative survival graph of patients.

3 risk factors were 107.1 ± 7.0, 78.0 ± 7.2, 61.1 ± 7.4 and 26.6 ± 10.3 mo, respectively. The 1, 5 and 10-year survival rates according to the number of risk factors were 96%, 87% and 87%, respectively, for patients with no risk factors (n = 29). For patients with 1 risk factor (n = 67), the corresponding rates were 89%, 65%, and 35%, respectively; and for patients with 2 risk factors (n = 25), the survival rates were 96%, 48%, and not available. For patients with 3 risk factors (n = 8), the 1-, 5- and 10-year survival rates were 63%, 17%, and 0%, respectively (P < 0.001; Figure 4).

The 1-year survival rates classified according to the Child-Pugh score (5 or ⩾ 6) were similar. Patients with a Child-Pugh score of 5 had 1-year survival rates of 96%, 94%, and 67% for patients with no, 1, and 2 risk factors, respectively; however, patients with a Child-Pugh score ⩾ 6 had 1-year survival rates of 92%, 94% and 62% for patients with no, 1, and 2 risk factors, respectively (Figure 5).

Response after TACE in patients with BCLC stage 0 and stage A HCC

Follow-up CT after the first TACE showed that the numbers of CR, PR, SD and PD were as follows: 4, 7, 107 and 0, respectively, according to the RECIST criteria; 99, 12, 4 and 3, respectively, according to the EASL criteria; and 100, 14, 3, and 2, respectively, according to the mRECIST criteria. Follow-up CT after the last TACE showed that the numbers of CR, PR, SD and PD were 16, 65, 16 and 10, respectively, according to the RECIST criteria; 40, 15, 17 and 40, respectively, according to the EASL criteria; and 49, 7, 7 and 44, respectively, according to the mRECIST criteria.

Forty-four patients (34.1%) had disease progression at the time of the follow-up CT scan. The average time to progression was 77.3 (5.1) mo, and the progression-free survival time was 33.8 (30.6) mo (Tables 4 and 5).

Table 3 Cox-regression analysis for risk factors of mortality

Variables	Cox-regression analysis		
	P value	HR	95%CI
Age (yr)	0.42	0.99	0.95-1.02
Gender (female/male[1])	0.008	3.47	1.39-8.68
Child-Pugh score (> 5/5[1])	0.005	3.86	1.50-9.91
Amount of lipiodol used (> 7 cc/⩽ 7 cc[1])	0.013	3.51	1.30-9.44
Arterio-venous shunt (yes/no[1])	0.032	4.41	1.14-17.11
Number of nodules (> 1/1[1])	0.530	1.63	0.36-7.51
Amount of doxorubicin used (> 27 cc/⩽ 27 cc[1])	0.318	0.98	0.93-1.02
BCLC class (A1/A0[1])	0.766	1.17	0.41-3.36
Lipiodol uptake (complete/incomplete[1])	0.174	2.47	0.67-9.08
Number of feeding vessel (⩾ 2/1[1])	0.086	0.32	0.09-1.17
Gelfoam use (yes/no[1])	0.054	2.38	0.98-5.76
Greatest diameter of nodule (cm)	0.395	0.85	0.57-1.25
AFP (ng/mL)	0.065	1.00	1.00-1.01

[1]Reference category. BCLC: Barcelona Clinic Liver Cancer; AFP: α-fetoprotein.

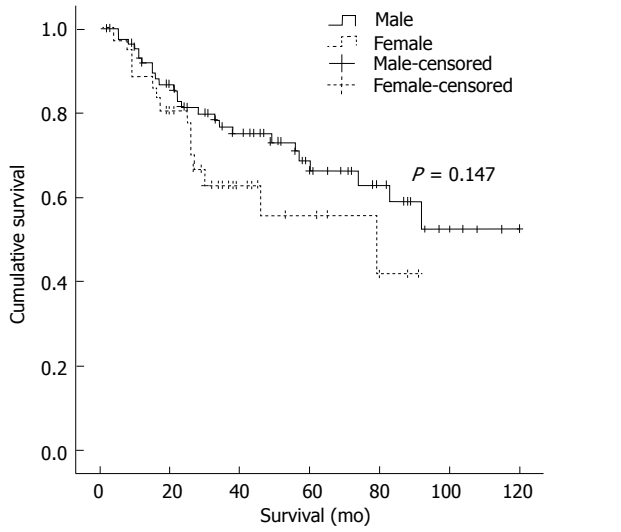
DISCUSSION

TACE is a widely used primary treatment for unresectable HCC and significantly delays tumor progression and vascular invasion[26]. For early-stage HCC, TACE is not indicated as a first-line option. In this study, which evaluated the efficacy of TACE as a first-line therapy for patients with BCLC stage 0 and stage A HCC, TACE had overall 1-, 5- and 10-year survival rates of 91%, 63% and 49%, respectively; these results are comparable to the results of other curative therapies (5-year survival of 50%-70%), including resection, transplantation, or percutaneous treatment[15,27,28]. Kinugasa *et al*[29] suggested that palliative TACE could be effective for treating HCC with 3 tumors or fewer (each up to 3 cm in diameter). Other data have also suggested that TACE provides a survival benefit for patients with early stage HCC[21,22]. Therefore, the efficacy of TACE in the treatment of BCLC stage 0 and stage A HCC might be comparable to that of other curative treatments.

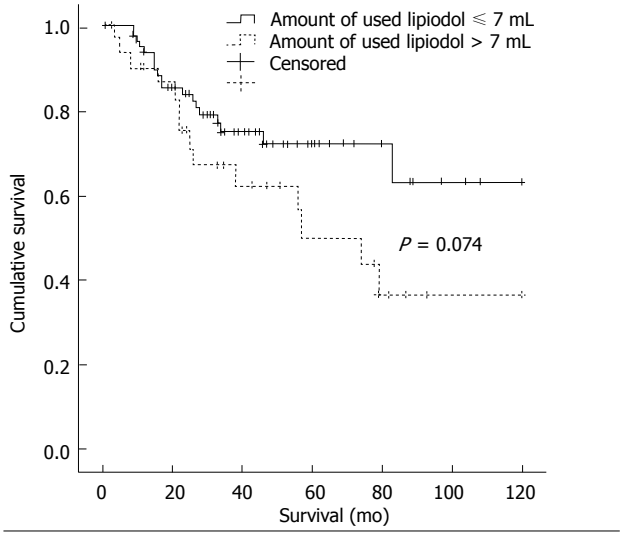
Our data also suggest that improvements in TACE technique, early diagnosis using precise imaging modalities, and a regular follow-up schedule according to standard guidelines could improve the survival rate of patients with BCLC stage 0 and stage A HCC.

In general, resection, transplantation, and ablation have been considered superior to TACE for very-early-stage or early-stage HCC. However, few studies comparing TACE and other curative therapies in patients with early-stage HCC are available. Therefore, prospective studies comparing the efficacy of TACE, surgery and ablation are needed in the future.

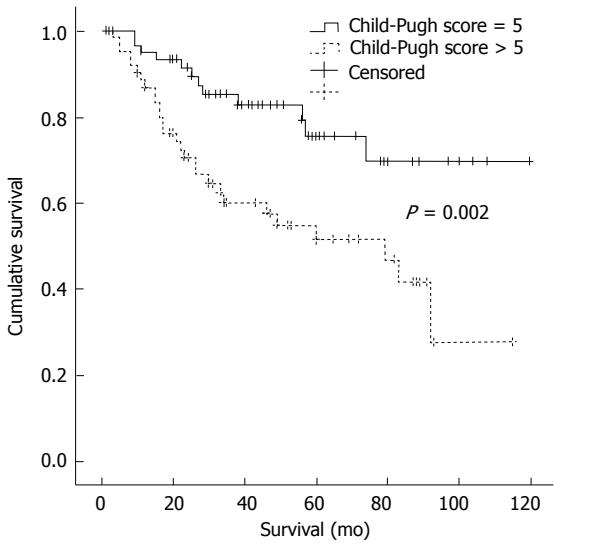
Although this study provides a rationale for the use of TACE in the treatment of patients with BCLC stage 0 and stage A HCC (particularly in patients in whom surgery or ablation is risky due to tumor location), further research comparing the efficacy of TACE with curative therapies is needed because these retrospective data do not provide enough evidence to fully support a potential survival benefit.



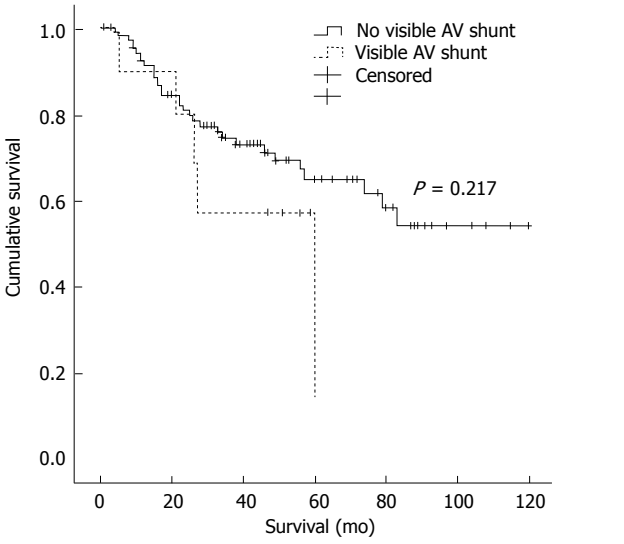
Survival	Mean (SE)	1-yr	5-yr	10-yr
Male	84.5 (5.6)	92%	66%	52%
Female	59.3 (6.4)	89%	56%	-



Survival	Mean (SE)	1-yr	5-yr	10-yr
Lipiodol ⩽ 7 mL	89.2 (6.2)	94%	72%	63%
Lipiodol > 7 mL	68.2 (9.2)	90%	50%	36%



Survival	Mean (SE)	1-yr	5-yr	10-yr
CPS = 5	95.8 (6.1)	95%	76%	70%
CPS ⩾ 6	64.4 (6.5)	87%	52%	28%



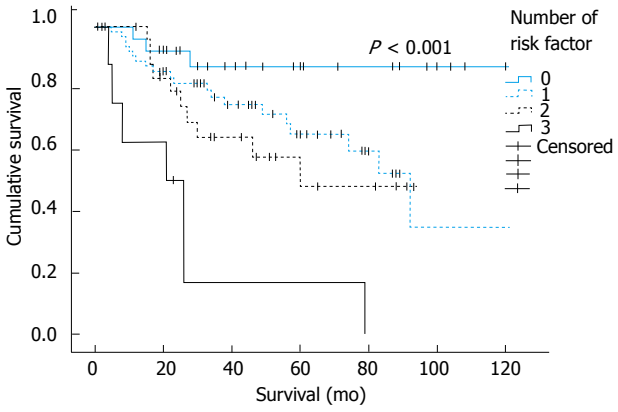
Survival	Mean (SE)	1-yr	5-yr	10-yr
No visible AV shunt	82.8 (5.3)	91%	65%	54%
Visible AV shunt	42.9 (7.5)	90%	0%	0%

Figure 3 Cumulative patient survival classified by each risk factor. CPS: Child-Pugh score; AV: Arterio-venous.

Previously reported risk factors for mortality following TACE in patients with intermediate or advanced HCC include the extent of lipiodol uptake, tumor location, number, and size, tumor marker levels, viral marker levels, patient age, and liver function[29-31]. The present study examined risk factors for mortality in patients with early-stage HCC and demonstrated that the Child-Pugh score, presence of arterio-venous shunt, amount of lipiodol used during TACE, and female gender were correlated with mortality.

The present study is the first report that discusses the outcome of TACE classified by the number of clinical risk factors among patients with BCLC stage 0 and stage A HCC. In the study, the number of risk factors was shown to be negatively correlated with survival. Overall, it seems likely that TACE could be a curative therapy for patients with fewer than 2 risk factors because our data showed that the 5-year survival rate in such patients was more than 50%. However, the results of this study suggest that TACE should remain a palliative treatment option for patients with ⩾ 2 risk factors; these patients should be treated with another curative-intent treatment following the first TACE.

Child-Pugh scores were a major risk factor for mortality in this study. Survival in patients with a Child-Pugh score of 5 was excellent (1, 5 and 10-year survival rates of 95%, 76% and 70%, respectively). Twenty-eight patients (70%) who died had a Child-Pugh score > 5. Sala *et al*[32]



Number of risk factor	Mean (SE)	1-yr	5-yr	10-yr
0	107.1 (7.0)	96%	87%	87%
1	78.0 (7.2)	89%	65%	35%
2	61.1 (7.4)	96%	48%	-
3	26.6 (10.3)	63%	17%	0%

Figure 4 Cumulative patient survival classified by the number of risk factors. Risk factors were Child-Pugh score > 5, presence of arterio-venous shunt, amount of lipiodol used > 7 mL during transarterial chemoembolization (TACE), and female gender.

reported that Child-Pugh class A was the strongest prognostic variable in patients undergoing percutaneous treatments. Shi *et al*[33] showed that the Child-Pugh class was significantly correlated with survival following TACE in patients with unresectable HCC. In addition, another study documented that the degree of Child-Pugh class was a risk factor for mortality[34]. However, among patients undergoing transplantation, the presence of a single HCC ≤ 5 cm or up to 3 nodules < 3 cm is an important risk factor for mortality[28]. Taken together, it can be concluded that the underlying Child-Pugh score is the primary risk factor for mortality following treatment of HCC, except in the case of transplantation.

TACE-related factors, including the presence of arterio-venous shunt and the amount of lipiodol used during TACE, were also important in predicting patient survival following the procedure. In early-stage HCC, the majority of the tumors are likely to be well differentiated and less invasive; as a result, such tumors can often be controlled by the complete obstruction of their blood supply. In theory, the presence of an arterio-venous shunt might cause the premature wash-out of gelatin sponge or lipiodol, eventually interrupting the complete obstruction of hepatic artery and the delivery of doxorubicin-based intra-arterial chemotherapy. Conversely, the amount of lipiodol used was positively correlated with tumor size, which is the key variable in staging systems[35]. A previous report demonstrated that heterogeneous lipiodol uptake was significantly correlated with local recurrence[29]. In addition, female gender was also a definite risk factor for mortality after TACE. Various factors might contribute to this result. By identifying these risk factors, an earlier prediction of overall survival may be possible, thereby improving the outcome of TACE by refining its delivery and technique.

Table 4 Response following transarterial chemoembolization in patients with Barcelona Clinic Liver Cancer stage 0 and stage A hepatocellular carcinoma

	CT findings after TACE											
	RECIST				EASL				mRECIST			
	CR	PR	SD	PD	CR	PR	SD	PD	CR	PR	SD	PD
1 mo after first TACE[1] (*n*)	4	7	107	0	99	12	4	3	100	14	3	2
1 mo after last TACE[2] (*n*)	16	65	16	10	40	15	17	40	49	7	7	44

[1]11 patients were excluded because of missing value; [2]22 patients were excluded because of missing value. TACE: Transarterial chemoembolization; RECIST: Response evaluation criteria in solid tumors; EASL: European Association for the Study of the Liver; m: Modified; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease.

Table 5 Recurrence, progression, and survival after transarterial chemoembolization

Numbers of patients with recurrence	77 (59.70%)
Numbers of patients with progression	44 (34.10%)
Time to recurrence and progression[1]	47.0 (4.6) and 77.3 (5.1)
1-yr recurrence and progression rate	33% and 22%
2-yr recurrence and progression rate	55% and 33%
3-yr recurrence and progression rate	61% and 39%
Recurrence-free survival[1]	27.8 (29.2)
Progression-free survival[1]	33.8 (30.6)

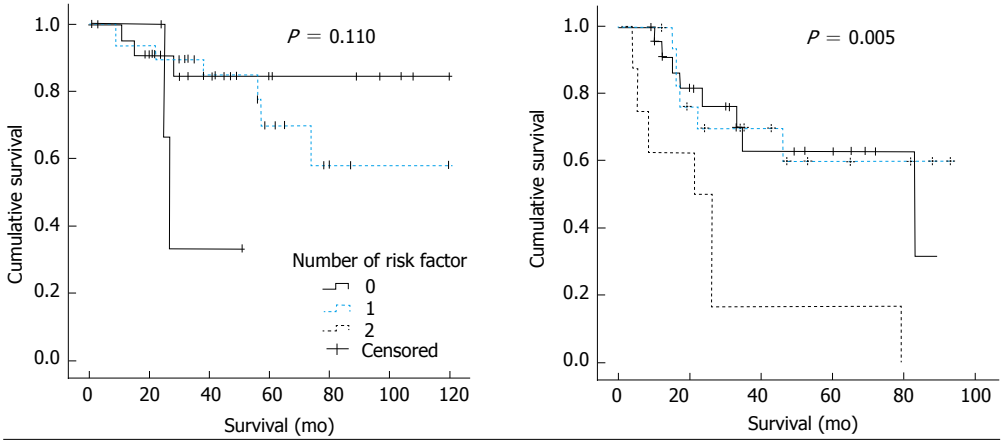
[1]months, mean (SD).

In this study, the RECIST criteria, in comparison with the EASL or mRECIST criteria, appeared to overlook many cases of complete response. Another study likewise demonstrated that RECIST often overlooked complete responses and mistakenly assessed the therapeutic efficacy of loco-regional therapies. Therefore, it is recommended that the response evaluation following TACE be estimated by the EASL or mRECIST criteria instead of the RECIST criteria.

A meta-analysis demonstrated that chemoembolization could improve the survival of carefully selected patients with unresectable HCC[36]. However, the efficacy of TACE for treating early stage HCC has not been thoroughly studied. In this study, time to progression was shown to be 77.3 ± 5.1 mo and progression-free survival time was 33.8 ± 30.6 mo. Forty-four (34.1%) patients in the study showed progression. The mean number of TACE procedures was 2.5 ± 2.1. Therefore, repeat TACE procedures based on surveillance imaging could increase progression-free survival in patients with BCLC stage 0 and BCLC stage A HCC.

The limitations of this study include the retrospective study design. Numerous other studies have reported on the TACE technique, including its advantages, methods, drug dosing, therapy protocol, scoring system, and cost[37-39]. Therefore, new approaches for reducing mortality in HCC should be available in the future.

In conclusion, TACE demonstrated a relatively good survival rate in patients with BCLC stage 0 and stage A



Number of risk factor	Child-Pugh score = 5			Child-Pugh score ⩾ 6		
	1-yr	5-yr	10-yr	1-yr	5-yr	10-yr
0	96%	84%	84%	92%	63%	-
1	94%	70%	58%	94%	60%	-
2	67%	-	-	62%	17%	-

Figure 5 One, 5, and 10-year survival rates classified by Child-Pugh score. Risk factors were Child-Pugh score > 5, presence of arterio-venous shunt, amount of lipiodol used > 7 mL during transarterial chemoembolization (TACE), and female gender.

HCC in this study. The Child-Pugh score, presence of arterio-venous shunt, amount of lipiodol used during TACE, and female gender were correlated with mortality in patients with BCLC stage 0 and stage A HCC who were treated with TACE. Patients with more than 2 risk factors should be treated by other curative-intent treatments after the first TACE.

COMMENTS

Background

In general, transarterial chemoembolization (TACE) has not been recommended as a first-line therapy for patients with Barcelona Clinic Liver Cancer (BCLC) stage 0 and stage A hepatocellular carcinoma (HCC), based on the results of a single retrospective study. However, this study analyzed the results of transarterial embolization, not TACE, and few studies comparing TACE with other curative therapies in patients with early stage HCC are available. Thus, considerable controversy remains regarding patient selection for TACE.

Innovations and breakthroughs

TACE demonstrated a relatively good survival rate in patients with BCLC stage 0 and stage A HCC in this study. The Child-Pugh score, presence of an arterio-venous shunt, amount of lipiodol used during TACE, and female gender were correlated with mortality in patients with BCLC stage 0 and stage A HCC who were treated with TACE.

Applications

Patients with more than 2 risk factors should be treated by other curative-intent treatments after the first TACE.

Terminology

According to RECIST, complete response (CR) is defined as the disappearance of all target lesions. Partial response (PR) is defined as a 30% minimum decrease in the sum of the longest diameter of target lesions, taking as reference the baseline sum of the longest diameter. Progressive disease (PD) is designated as a 20% minimum increase in the sum of the longest diameter of target lesions, taking as reference either the smallest sum of the longest diameter recorded since the beginning of treatment or the appearance of one or more new lesions. Stable disease (SD) is indicated by insufficient shrinkage to qualify for partial response. According to the European Association for the Study of the Liver guidelines, CR is defined as the absence of enhancing tumor areas, reflecting complete tissue necrosis. PR is defined as a > 50% decrease in the enhancing areas, reflecting partial tissue necrosis. PD is defined as a > 25% increase in the size of a single, measurable lesion or as the appearance of new lesions. SD is defined as a tumor response classified between PR and PD. The response categories, according to the criteria of mRECIST, are defined as follows. CR is defined as the disappearance of any intra-tumoral arterial enhancement in all target lesions; PR is defined as at least a 30% decrease in the sum of the diameters of viable target lesions; SD is defined as any cases that do not qualify for either PR or PD; and PD is defined as an increase of at least 20% in the sum of the diameters of viable target lesions.

Peer review

In this paper, the authors retrospectively investigate the effect of TACE on early stage HCC survival. Because the role of TACE in this setting is still under debate, this paper is of interest to the readers of the Journal. The sample size is adequate, and the methods are appropriately chosen. A 10-year retrospective study design was used. If promising results were obtained, the clinical importance is potentially significant.

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ORIGINAL ARTICLE

Epidermal growth factor upregulates Skp2/Cks1 and p27^{kip1} in human extrahepatic cholangiocarcinoma cells

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Abstract

AIM: To evaluate the expression status of S-phase kinase-associated protein 2 (Skp2)/cyclin-dependent kinases regulatory subunit 1 (Cks1) and p27^{kip1}, and assess the prognostic significance of Skp2/Cks1 expression with p27^{kip1} in patients with extrahepatic cholangiocarcinoma.

METHODS: Seventy-six patients who underwent curative resection for histologically confirmed extrahepatic cholangiocarcinoma at our institution from December 1994 to March 2008 were enrolled. Immunohistochemical staining for Skp2, Cks1, p27^{kip1}, and Ki67, along with other relevant molecular biologic experiments, were performed.

RESULTS: By Cox regression analyses, advanced age (> 65 years), advanced AJCC tumor stage, poorly differentiated histology, and higher immunostaining intensity of Skp2 were identified as independent prognostic factors in patients with extrahepatic cholangiocarcinoma. Exogenous epidermal growth factor (EGF, especially 0.1-10 ng/mL) significantly increased the proliferation indices by MTT assay and the mRNA levels of Skp2/Cks1 and p27^{kip1} in SNU-1196, SNU-1079, and SNU-245 cells. The protein levels of Skp2/Cks1 (from nuclear lysates) and p27^{kip1} (from cytosolic lysate) were also significantly increased in these cells. There were significant reductions in the protein levels of Skp2/Cks1 and p27^{kip1} (from nuclear lysate) after the treatment of LY294002. By chromatin immunoprecipitation assay, we found that E2F1 transcription factor directly binds to the promoter site of *Skp2*.

CONCLUSION: Higher immunostaining intensity of Skp2/Cks1 was an independent prognostic factor for patients with extrahepatic cholangiocarcinoma. EGF upregulates the mRNA and protein levels of Skp2/Cks1 and p27^{kip1} *via* the PI3K/Akt pathway and direct binding of E2F1 transcription factor with the *Skp2* promoter.

Key words: S-phase kinase-associated protein 2; Cyclin-dependent kinases regulatory subunit 1; P27^{kip1}; Cholangiocarcinoma; E2F1; PI3K/Akt

Core tip: Based on the idea that S-phase kinase-associated protein 2 (Skp2) and p27^{kip1} might play a role in the pathogenesis and disease progression of patients with extrahepatic cholangiocarcinoma, immunohistochemical staining for Skp2, cyclin-dependent kinases regulatory subunit 1 (Cks1), p27^{kip1}, and Ki67, along with other relevant molecular biologic experiments, were performed in tissue samples and human cholangiocarcinoma cell lines. Higher immunostaining intensity of Skp2 was an independent prognostic factor in patients with extrahepatic cholangiocarcinoma, and exogenous epidermal growth factor upregulates mRNA and protein levels of Skp2/Cks1 and p27^{kip1} in SNU-1196, SNU-1079, and SNU-245 cells. By chromatin immunoprecipitation assay, we found that E2F1 transcription factor directly binds to the promoter site of *Skp2*.

Kim J, Kim HJ, Park JH, Park DI, Cho YK, Sohn CI, Jeon WK, Kim BI, Kim DH, Chae SW, Sohn JH. Epidermal growth factor upregulates Skp2/Cks1 and p27^{kip1} in human extrahepatic cholangiocarcinoma cells. *World J Gastroenterol* 2014; 20(3): 755-773 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i3/755.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i3.755

INTRODUCTION

Human extrahepatic cholangiocarcinoma (CC) is a highly malignant epithelial cancer of the biliary tract with high morbidity and mortality. Although certain conditions are associated with greater risk (*i.e.*, sclerosing cholangitis[1] and hepatolithiasis[2,3]), most cases are sporadic. Despite the current knowledge of etiology and pathology of CC, its cellular and molecular pathogenesis is still poorly understood.

Ubiquitin-directed protein degradation plays an important role in regulating a broad spectrum of biologic processes, including transcription, signal transduction, cell cycle progression, apoptosis, cell growth, differentiation, and development. Proteins that are targeted for degradation by this mechanism undergo a series of ubiquitin-modification steps carries out by the successive activity of E1 (activating), E2 (conjugating), and E3 (ligating) ubiquitin enzymes[4,5]. SCF (Skp, Cullin, F-box containing complex) complexes belong to a large family of multi-subunit E3 ubiquitin ligases that select and ubiquitinate specific proteins for targeted destruction by the 26S proteasome.

S-phase kinase associated protein 2 (Skp2) is the substrate-recognition subunit of the SCF^{skp2} E3 ligase complex. Several lines of evidence from both biochemical and genetic studies have shown that Skp2 is required for cell cycle progression at multiple stages, including G1/S transition, S phase progression, and S/G2 transition[6-8]. A principal substrate responsible for these activities of Skp2 in the cell cycle phases is p27^{kip1}, an inhibitor of cdk2 and cdk1 activities that promote entry into DNA synthesis and mitosis, respectively[8-10]. The important role of Skp2 in promoting entry into the S phase, mainly by promoting p27^{kip1} destruction, was observed in cell-free systems, cell cultures, and in animal models[9-12]. Those findings raised the question of whether the low levels of p27^{kip1} in human cancers are caused by increased expression of Skp2.

Cyclin kinase subunit 1 (Cks1) is an additional protein that has a role in efficient interaction between the Skp2 ubiquitin ligase complex and its substrate p27^{kip1}. The critical role of Cks1 in targeting p27^{kip1} was indicated in the studies which demonstrated the lack of p27^{kip1} ubiquitylation and breakdown in the absence of Cks1 *in vitro* in cell-free systems[13] and the slow cell proliferation and accumulation of p27^{kip1} in Cks1 nullizygous mice *in vivo*[14]. The expression of Cks1 mRNA and protein levels is strongly correlated with the expression of Skp2 protein levels and inversely with those of p27^{kip1} levels in colorectal, breast, gastric, prostate, oral epithelial, and non-small cell lung cancers[15-21]. In some of these cancers, including breast, colorectal, and gastric cancers, Skp2 and Cks1 expression were independent prognostic markers and provided additional prognostic information in these cancer patients[22-25].

To our knowledge, there have been no reports concerning the expression status and the prognostic implication of Skp2 and Cks1 in extrahepatic cholangiocarcinoma. The overall objective of our study is to evaluate the expression status of Skp2/Cks1 and p27^{kip1}, and assess the significance of Skp2/Cks1 expression with p27^{kip1} as a predictive prognostic marker in patients with extrahepatic cholangiocarcinoma. Additionally, we explored the molecular biologic mechanisms for the overexpression of Skp2/Cks1 and down-regulation of p27^{kip1} using the intrahepatic and extrahepatic cholangiocarcinoma cell lines.

MATERIALS AND METHODS

Patients

Archived paraffin embedded tissue blocks of 76 cases of extrahepatic cholangiocarcinoma who underwent curative surgery at Kangbuk Samsung Hospital from January 1994 to March 2008 were included in our study. Medical records and laboratory data of each patient were retrospectively reviewed. Survival data (including overall and recurrence free survival) were collected by review of medical records and contact with patients or their relatives by telephone interview. This study was conducted in accordance with the principles of the Declaration of Helsinki. Our study protocol was approved by the Ethics Committee of Kangbuk Samsung Hospital (C0653, approved at 2009-4-23).

Materials

The following primary or secondary antibodies were used: anti-Skp2 (Invitrogen, 32-3300), anti-p27^{kip1} (BD Biosciences, 610241), anti-Cks1 (Invitrogen, 36-6800),

anti-Ki-67 (Dako), monoclonal mouse anti-E2F1 (Abcam, ab483), and monoclonal mouse anti-β-actin (Abcam, ab8226). PI3K inhibitor LY294002 and mitogen activated protein kinase (MAPK) inhibitor PD169316 were purchased from Calbiochem.

Immunohistochemistry

Immunohistochemical staining for Skp2, Cks1, p27^{kip1}, and Ki67 were performed using the relevant microarray tissue blocks of cholangiocarcinoma. The tissue sections (4 μm) were cut, placed on silane-pretreated slides, deparaffinized, and rehydrated through graded alcohols. Antigen retrieval was performed by microwave heating at high power (750 W) in 10 mmol/L sodium citrate buffer (pH = 6) for 4 cycles of 5 min each. Slides were then allowed to cool for 30 min prior to incubation with either the primary antibodies targeting Skp2 (2C8D9, 1:100, Zymed), Cks1 (4G12G7, 1:250, Zymed), p27^{kip1} (1B4, 1:20, Zymed), or Ki-67 (MIB-1, 1:100, DAKO) for 1 h at room temperature. Staining was performed with the Envision Monoclonal System (Dako, Carpinteria, CA, United Sates). The reaction was developed with DAB. Slides were counterstained with Mayer's hematoxylin and mounted. Incubation without the primary antibody was used as a negative control.

Two pathologists (Sohn JH and Chae SW) blinded to the follow-up data independently evaluated the immunohistochemically stained slides. If there were discrepancies between them, the results were read again by both pathologists together. The staining intensities of Skp2, Cks1, and p27^{kip1} were semi-quantitatively measured at × 400 magnification, and staining intensity of the nucleus and cytoplasm was independently categorized as no staining (intensity = 0), weak (intensity = 1), moderate (intensity = 2), or strong (intensity = 3, Figure 1). The number of staining positive cells was expressed as a percentage of the total number of epithelial cells and assigned to the one of five categories: score 0, < 5%; score 1, 5%-25%; score 2, 26%-50%; score 3, 51%-75%; score 4, > 75%. The percentage score of staining positive tumor cells and the staining intensity were multiplied to produce an immunoreactive score (IS) for each tumor specimen. Ki-67 immunostaining results (Ki-67 LI) were recorded as the percentage of immunoreactive cells over at least 2000 tumor cells randomly selected from the periphery of each of the invasive carcinoma in the surgical specimens. The slides were examined under a Leica DMR microscope and images were captured using a Leica digital camera (Leica Microsystems Inc., 1700 Leider Lane, Buffalo Grove, IL, United States). Immunoreactive cells were counted randomly with a minimum of 20 high-power fields (× 400), with the help of an image computer analyzer (ImageJ, Image Processing and Analysis in Java).

Cell lines and culture

Human intrahepatic duct cancer cells SNU-1079 (KCLB No. 01079), human hepatic duct bifurcation site cancer cells SNU-1196 (KCLB No. 01196), and human common bile duct cancer cells SNU-245 (KCLB No. 00245) were maintained in RPMI1640 medium supplemented with L-glutamine (300 mg/L), 25 mmol/L HEPES, 25 mmol/L $NaHCO_3$ (90%), and 10 % heat inactivated fetal bovine serum[26].

Cell proliferation assay

A total of 3 × 10^3 cells were seeded into 96-well plates and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, M5655, Sigma, St. Louis, MO, United States] was added to each well every 24 h. The plates were incubated at 37 ℃ for 4 h followed by addition of 100 μL lysate (10% SDS in 0.01 mol/L HCl). The absorbance was measured at 490 nm using an ELISA microplate reader.

Quantitative polymerase chain reaction

Total RNA was extracted from the cells using the TRIzol Reagent (Invitrogen), and was reverse-transcribed into complementary DNA with the Takara RNA PCR Kit (TAKARA Bio Inc., Otsu, Shiga, Japan). Quantification of the complementary DNA template was performed with a quantitative polymerase chain reaction (qPCR, T3000 thermocycler, Biometra GmbH, Goettingen, Germany) using Thermocycler Manager Software (V4.11, Biometra GmbH, Goettingen, Germany). PCR primers are as follows: Skp2: forward, 5'-CCAGCAAGACTTCTGAAC-3', reverse, 5'-GGAGGCACAGACAGGAAA-3'; p27^{kip1}: forward, 5'-GCAACCGACGATTCTTCTAC-3', reverse, 5'-GTCCATTCCATGAAGTCAGC-3'; Cks1: forward, 5'-CCCACTACCCAAGAAACCAA-3', reverse, 5'-CCGCAAGTCACCACACATAC-3'; E2F1 forward: 5'-CGTGAGCGTCATGGCCTTGG-3', reverse: 5'-GGCGTCCCTGGGGTCCGTAC-3'; β-actin forward: 5'-CAAGAGATGGCCACGGCTGCT-3', reverse: 5'-TCCTTCTGCATCCTGTCGGCA-3'.

Immunoblot analysis

Various concentration of exogenous EGF (0.01-100 ng/mL) were added onto cultured tumor cell lines. Cells were directly lysed for 30 min on ice with lysis buffer [50 mmol/L Tris-HCl (pH = 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L PMSF, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1 mmol/L Na_3VO_4, and 1 mmol/L NaF). After centrifugation at 13000 *g* for 15 min, protein concentrations were measured using Bradford's reagent (Bio-Rad, Hercules, CA, United States), and protein was denatured by boiling for 10 min. Protein (25 μg) was loaded onto 10% SDS-PAGE and then transferred onto nitrocellulose membranes. After blocking with 5% milk in TBST (137 mmol/L NaCl, 25 mmol/L Tris and 1 mmol/L disodium ethylenediaminetetraacetate containing 0.1% Tween-20), the membranes were incubated with appropriate primary antibodies at a dilution of 1:1000 at 4 ℃ overnight. After washing with TBST three times (each for 10 min), the membranes were incubated with their corresponding horseradish peroxidase-

conjugated secondary antibodies at a dilution of 1:1000 at room temperature for 1 h. After washing with TBST three times (each for 10 min), bound antibodies were visualized using enhanced chemiluminescent substrates (Amersham, Arlington Heights, IL, United States). For nuclear and cytoplasmic extracts, cells were processed using the Thermo Scientific NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific Inc. Rockford, IL, United States), according to the manufacturer's instructions.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assay was performed using a commercially available assay kit (#17-229, Upstate Biotechnology, Lake Placid, NY, United States) according to the manufacturer's instructions. Cells were briefly incubated with 1% formaldehyde in media at 37 ℃ for 15 mitosis cross-link DNA and DNA-binding proteins. After washing in PBS, cells were collected and lysed within the 200 μL sodium dodecyl sulfate lysis buffer supplied by the kit. The cell extracts were sonicated with four sets of 10-s pulses using a sonicator (Sonifier cell disruptor; Heat Systems Ultrasonics, Plainview, NY, United States) equipped with a 2-mm tip and set to 30% of maximum power to shear the DNA into 200- to 1000-bp fragments, and then centrifuged at 13000 *g* for 10 min. The DNA concentration in the supernatant was quantitated by measuring absorbance at 260 nm. Samples containing 200 μg DNA was diluted with the chromatin immunoprecipitation dilution buffer to a final volume of 2050 μL. Fifty microliters from each sample was removed as a non-immunoprecipitation input control. After preclearing the sample with 75 μL salmon sperm DNA/protein A agarose slurry at 4 ℃ for 30 min, the target protein E2F1 was immunoprecipitated with 2 μg anti-E2F1 monoclonal antibody at 4 ℃ overnight with rotation using a multipurpose rotator (Model 151; Scientific Industries, Bohemia, NY, United States). A mock immunoprecipitation without antibody was also performed. Next, 60 μL of the salmon sperm DNA/protein A agarose slurry was added and incubated at 4 ℃ for 60 min. After washing the agarose beads, the protein A-agarose immune complexes were eluted, in two separate 250-μL aliquots, in elution buffer (1% SDS, 0.1 mol/L $NaHCO_3$) at room temperature for 30 min. Twenty-five microliters of each eluent was used for immunoblot analysis to determine the immunoprecipitation efficiency. Following the addition of 5 mol/L NaCl (20 μL), protein-DNA cross-linking was reversed by heating at 65 ℃ for 5 h. After digestion with 2 μL of 10 mg/mL proteinase K at 45 ℃ for 1 h, DNA fragments were purified *via* phenol/chloroform extraction and ethanol precipitation, and the DNA pellet was dissolved in 20 μL water. The following PCR primers were employed to amplify the 238-bp product spanning the E2F1-specific binding site identified in the 5'-flanking region of the human *Skp2* gene: forward, 5'-GAGAGAGACAGGGCAATCATACAC-3'; reverse, 5'-ATCACCAGAAGGCCTGCGGGCT-3'. The copy number for amplified PCR products was quantitated using qPCR as described in the qPCR section.

Statistical analysis

Continuous variables were compared by Student's *t* test and one-way ANOVA. Categorical variables were evaluated using the χ^2 or two-tailed Fisher exact test. For comparison of overall survival between the patient group with each immunohistochemical staining intensity and IS, Kaplan-Meier analyses with Log-rank comparisons were performed. Multivariate logistic regression analysis was performed to identify independent prognostic factors. All tests were 2-sided, and a *P* value of less than 0.05 was considered statistically significant. Statistical analysis was conducted using the PASW statistics package 18 (IBM, Armonk, New York, NY, United States).

RESULTS

Clinicopathologic characteristics and immunohistochemical expressions of Skp2, Cks1, Ki-67, and p27^{kip1}

The baseline clinicopathologic characteristics of the 76 patients with extrahepatic cholangiocarcinoma enrolled in the current study are listed in Table 1. Immunohistochemical staining showed that Skp2, Cks1, and Ki67 proteins were expressed in the nuclei of cancer cells (Figure 1A-C). The staining pattern of p27^{kip1} was somewhat heterogeneous. The pattern of nuclear staining was most noticeable, some of which were expressed as diffuse cytoplasmic staining (Figure 1D). The results of the immunohistochemical staining for Skp2, Cks1, Ki67, and p27^{kip1} were as follows. Skp2 intensity: 0, 32 (42.1%); 1, 21 (27.6%); 2, 16 (21.1%); 3, 7 (9.2%); Cks1 intensity: 0, 9 (11.8%); 1, 24 (31.6%); 2, 37 (48.7%); 3, 6 (7.9%); p27^{kip1} intensity: 0, 23 (30.3%); 1, 31 (40.8%); 2, 15 (19.7%); 3, 7 (9.2%); Ki67: negative, 33 (43.4%); positive, 43 (56.6%).

The staining intensity of Skp2 and Cks1 showed significant linear correlation (Pearson's $r = 0.427$, $P < 0.01$). The staining intensity of Skp2 and Cks1 also showed significant linear correlations with the staining intensity of p27^{kip1} (Pearson's $r = 0.314$, $P < 0.01$ for Skp2 and p27^{kip1}; Pearson's $r = 0.403$, $P < 0.01$ for Cks1 and p27^{kip1}). The Ki-67 labeling index (LI) significantly increased as the staining intensity of Skp2 and Cks1 increased ($P < 0.01$). However, the staining intensity of p27^{kip1} did not correlate with the Ki-67 LI (Figure 2).

By Kaplan-Meier analyses, a tumor size larger than 2 cm ($P < 0.01$, Figure 3A), advanced AJCC stage ($P < 0.01$, Figure 3B), moderately and poorly differentiated histology ($P < 0.01$, Figure 3C), R1 resection ($P < 0.01$, Figure 3D), presence of angiolymphatic tumor invasion ($P = 0.036$, Figure 3E), and stronger immunostaining intensity of Skp2 ($P < 0.01$, Figure 3F) were significantly associated with shorter survival for patients with extrahepatic cholangiocarcinoma.

To explore the independent and significant prognostic factors for patients with extrahepatic cholangiocarcino-

Table 1 Clinicopathologic characteristics and immunohistochemical expressions in 76 patients with extrahepatic cholangiocarcinoma

Parameters		*n* (%)
Sex	Male	47 (61.8)
	Female	29 (38.2)
Age	mean ± SD	63.1 ± 9.3
Surgical resection	CBD segmental Resection	32 (42.1)
	Whipple or PPPD	44 (57.9)
Tumor size	⩾ 2 cm	48 (63.2)
	< 2 cm	28 (36.8)
American Joint Commission on Cancer stage	Ⅰ A	10 (13.2)
	Ⅰ B	18 (23.7)
	Ⅱ A	20 (26.3)
	Ⅱ B	26 (34.2)
	Ⅲ	1 (1.3)
	Ⅳ	1 (1.3)
Histologic type	Well-differentiated	19 (25.0)
	Moderately-differentiated	46 (60.5)
	Poorly-differentiated	11 (14.5)
Resection margin	R0	57 (75.0)
	R1	19 (25.0)
Angiolymphatic invasion	Absent	31 (40.8)
	Present	45 (59.2)
Perineural invasion	Absent	36 (47.4)
	Present	40 (52.6)
Regional lymph nodes involvement	Absent	49 (64.5)
	Present	27 (35.5)
Skp2 immunostaining	Intensity	
	0	32 (42.1)
	1	21 (27.6)
	2	16 (21.1)
	3	7 (9.2)
	Immunoreactive score	
	0	43 (56.6)
	1	10 (13.2)
	2	15 (19.7)
	3	6 (7.9)
	6	2 (2.6)
Cyclin-dependent kinases regulatory subunit 1 immunostaining	Intensity	
	0	9 (11.8)
	1	24 (31.6)
	2	37 (48.7)
	3	6 (7.9)
	Immunoreactive score	
	0	24 (31.6)
	1	12 (15.8)
	2	26 (34.2)
	3	1 (1.3)
	4	8 (10.5)
	6	4 (5.3)
	9	1 (1.3)
p27^{kip1} immunostaining	Intensity	
	0	23 (30.3)
	1	31 (40.8)
	2	15 (19.7)
	3	7 (9.2)
	Immunoreactive score	
	0	29 (38.2)
	1	22 (28.9)
	2	6 (7.9)
	3	2 (2.6)
	4	8 (10.5)
	6	7 (9.2)
	9	2 (2.6)
Ki-67 LI	mean ± SD	4.0 ± 6.7

CBD: Common bile duct; LI: Labeling index; PPPD: Pylorus preserving pancreatoduodenectomy; Skp2: S-phase kinase-associated protein 2.

ma, we performed Cox regression analyses with the forward stepwise conditional method. As a result, advanced age (> 65 years), advanced AJCC tumor stage, histologic type of tumor, and immunostaining intensity of Skp2 were identified as independent and significant prognostic factors for patients with extrahepatic cholangiocarcinoma (Table 2).

Exogenous EGF increased the proliferation indices, mRNA, and protein levels of SKP2/Cks1 and p27^{kip1} in SNU-1196, SNU-1079, and SNU-245 cells

qPCR and western blotting for Skp2/Cks1 and p27^{kip1} after the exogenous treatment of variable concentrations of EGF into the cell culture mediums were subsequently performed. Exogenous EGF (especially 0.1-10 ng/mL) significantly increased the proliferation indices of SNU-1196, SNU-1079, and SNU-245 cells by MTT assay (Figure 4).

Exogenous EGF (especially 0.1-10 ng/mL) significantly upregulated the mRNA levels of SKP2/Cks1 and p27^{kip1} in SNU-1196 (Figure 5A), SNU-1079 (Figure 5B), and SNU-245 cells (Figure 5C).

The protein levels of SKP2/Cks1 (from nuclear lysates) and p27^{kip1} (from cytosolic lysate) were significantly increased in SNU-1196 (Figure 6A), SNU-1079 (Figure 6B), and SNU-245 cells (Figure 6C) after exogenous treatment of variable concentrations of EGF into the cell culture mediums.

Protein levels of SKP2/Cks1 were dependent upon PI3K/Akt pathway

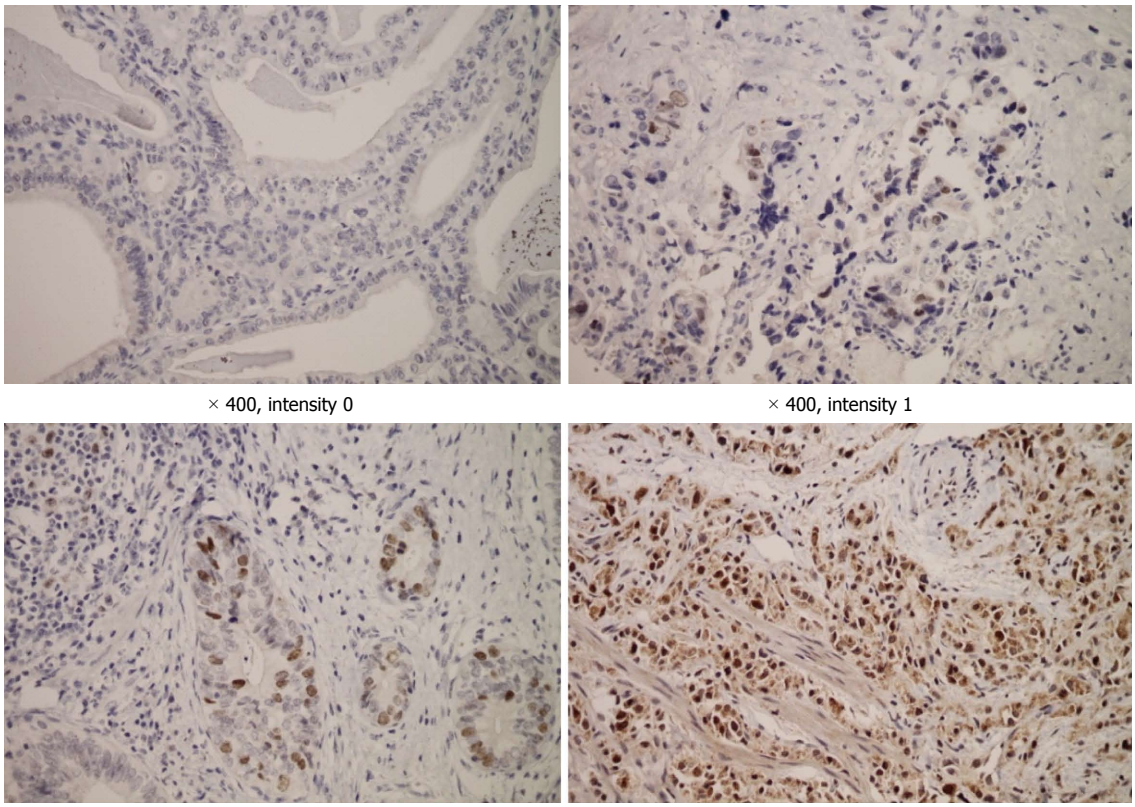
To explore the intracellular signal transduction pathways responsible for the upregulation of the mRNA and protein levels of Skp2/Cks1, we treated the 3 cholangiocarcinoma cell lines with LY294002 (PI3K inhibitor, 5 μmol/L) and PD0325901 (MEK1/2 inhibitor, 100 nmol/L). There were significant reductions in the protein levels of SKP2/Cks1 and p27^{kip1} (from nuclear lysate) after the treatment of LY294002. However, there were no significant changes in the protein levels of SKP2/Cks1 and p27^{kip1} (from nuclear lysate) after the treatment of PD0325901 (Figure 7).

E2F1 transcription factor binds to the promoter region of Skp2 gene

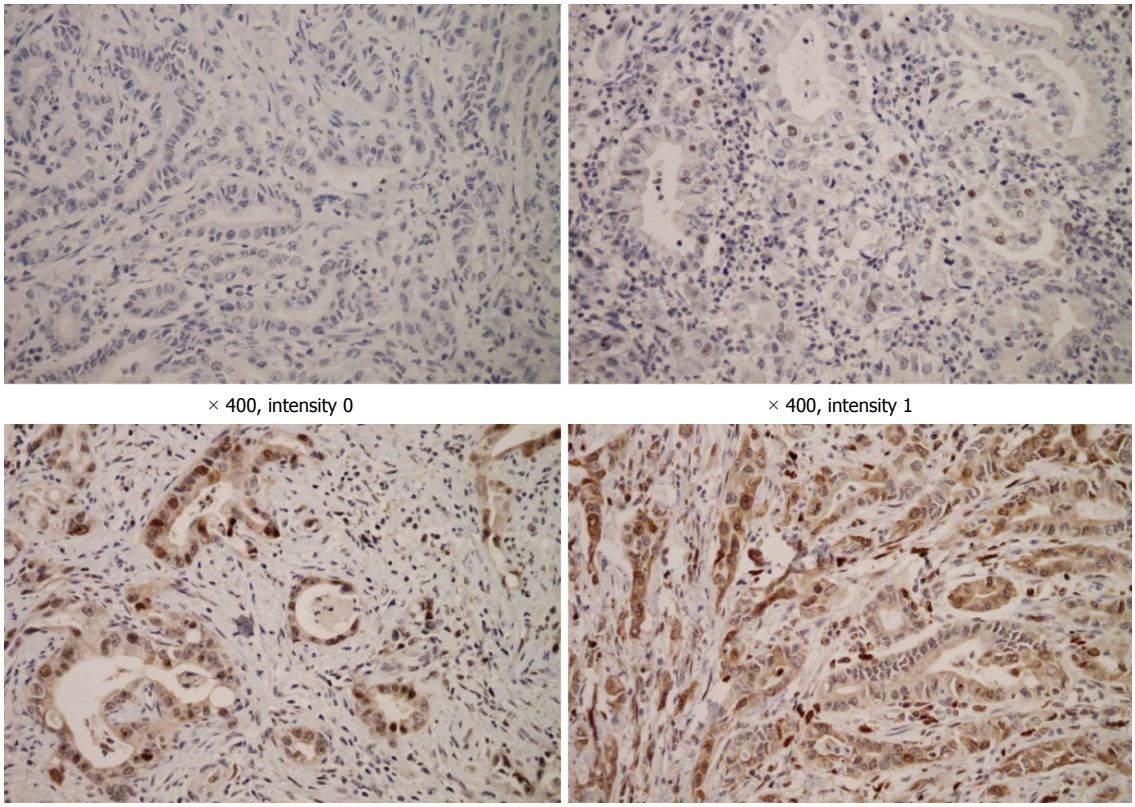
Because the PI3K signaling pathway regulates *Skp2* transcription and the molecular mechanism linking PI3K signaling to the *Skp2* gene is unclear, we investigated *Skp2* regulation more precisely. To show transcriptional control of Skp2 after the addition of 10 ng/mL of EGF, we did chromatin immunoprecipitation (ChIP) assays. As shown in Figure 8, E2F1 transcription factor directly binds to the promoter site of *Skp2*.

DISCUSSION

Skp2 and Cks1, the ubiquitin ligase subunits that target p27^{kip1} for degradation, are commonly overexpressed in


A
× 400, intensity 0
× 400, intensity 1
× 400, intensity 2
× 400, intensity 3

Skp2


B
× 400, intensity 0
× 400, intensity 1
× 400, intensity 2
× 400, intensity 3

Cks1

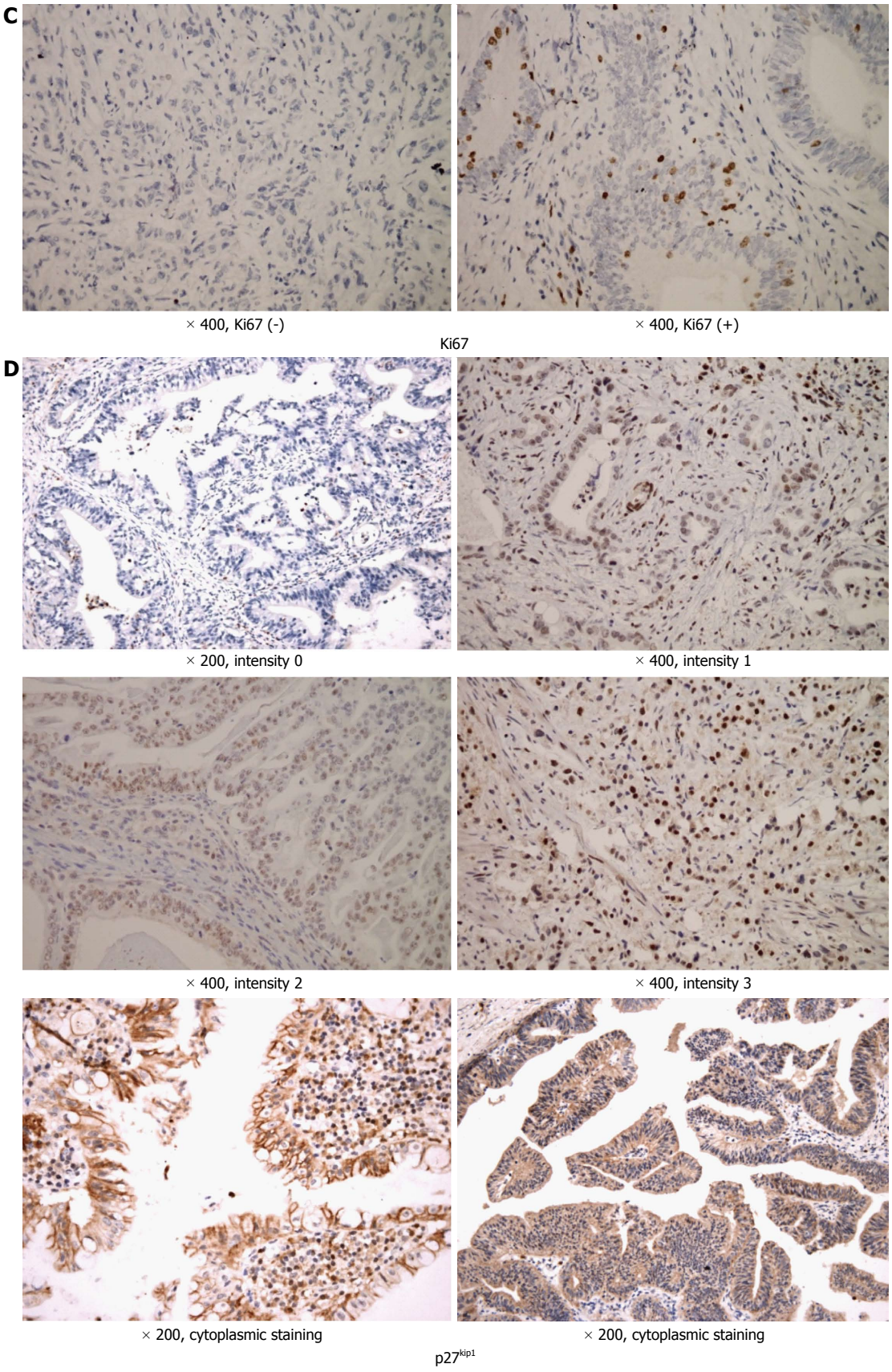


Figure 1 Results of immunohistochemical staining. A-C: Immunohistochemistry for (A) Skp2, (B) Cks1, and (C) Ki67 proteins showed that stains were mainly expressed in the nuclei of cancer cells; D: The staining pattern of p27^{kip1} was somewhat heterogeneous. The pattern of nuclear staining was mainly noticed, and some of them were expressed as diffuse cytoplasmic staining. The staining intensity of Skp2, Cks1, and p27^{kip1} was classified as follows: 0, no staining; 1, weak; 2, moderate; 3, strong. The staining pattern of Ki-67 was nuclear and classified as positive or negative. At least 20 high-power fields will be randomly chosen, and 2000 cells will be always counted. Skp2: S-phase kinase-associated protein 2; Cks1: Cyclin-dependent kinases regulatory subunit 1.

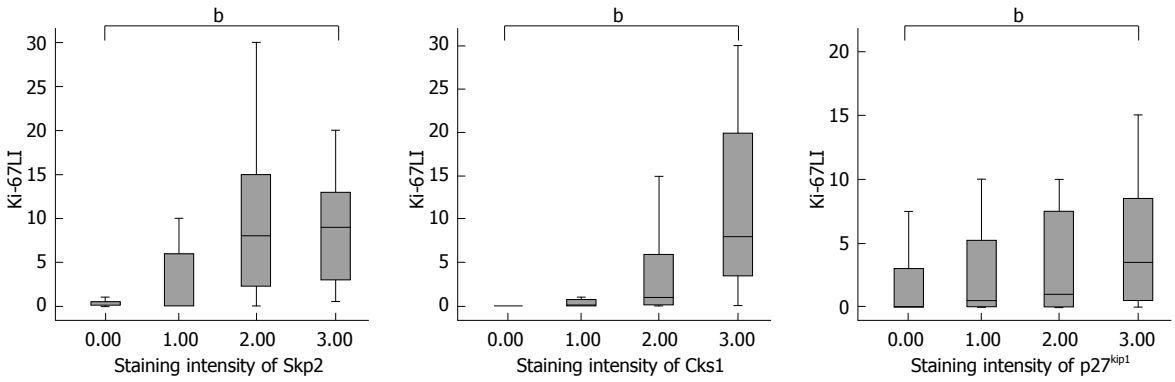


Figure 2 Relationship among the immunostaining intensity for S-phase kinase-associated protein 2, Cyclin-dependent kinases regulatory subunit 1, p27^{kip1}, and Ki-67 LI. Ki-67 LI significantly increased as the staining intensity of Skp2 and Cks1 increased. Ki-67 LI showed no significant correlation with the staining intensity of p27^{kip1}. Statistical analyses were performed by one-way ANOVA. $^{b}P < 0.01$ between groups. Skp2: S-phase kinase-associated protein 2; Cks1: Cyclin-dependent kinases regulatory subunit 1.

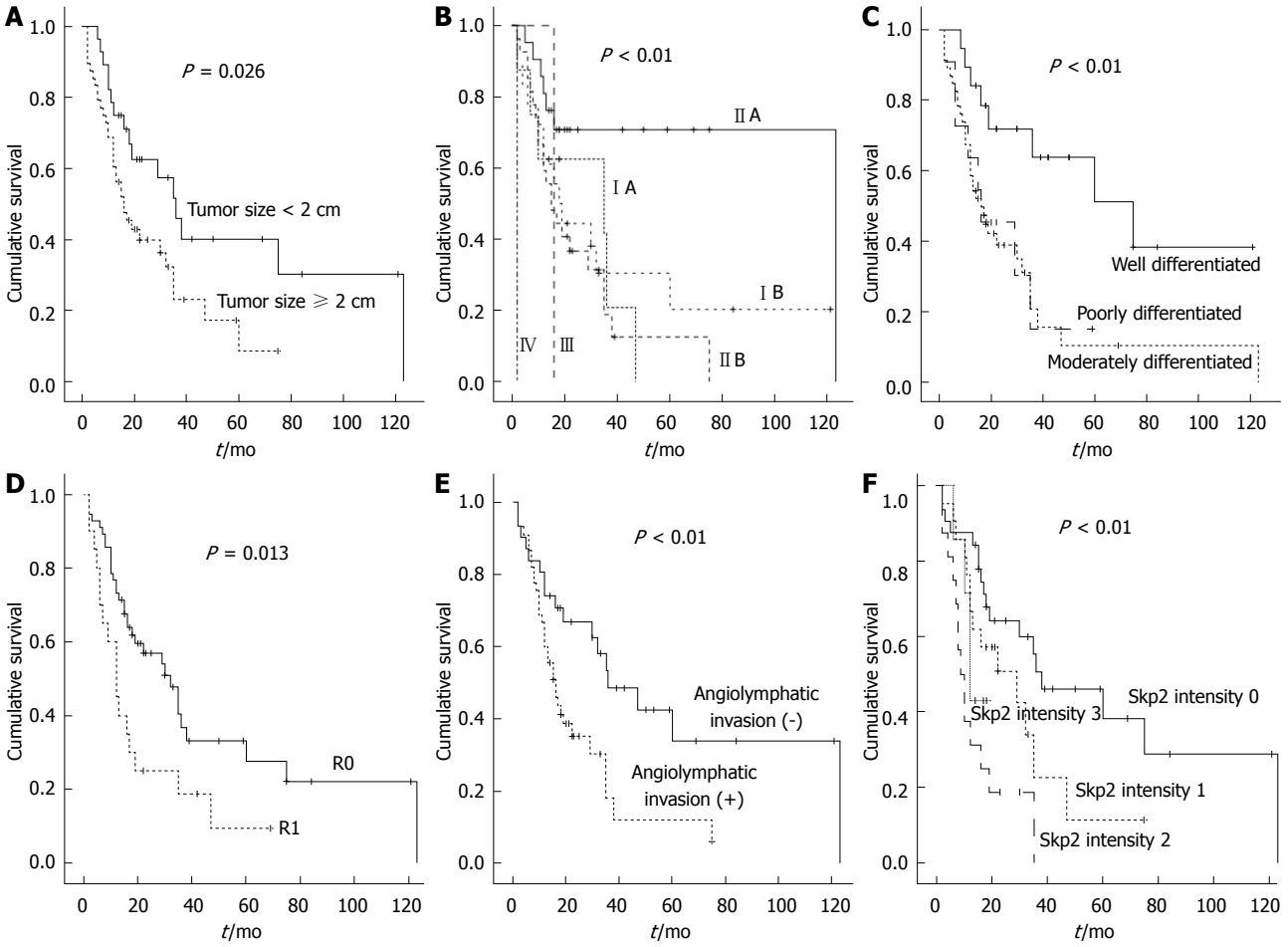


Figure 3 Results of Kaplan-Meier survival analysis. By Kaplan-Meier survival analysis, tumor size larger than 2 cm (A), advanced American Joint Commission on Cancer stage (B), tumor differentiation (C), R1 resection (D), angiolymphatic tumor invasion (E), and the intensity of Skp2 immunostaining (F) were significantly associated with overall survival in patients with extrahepatic cholangiocarcinoma. Skp2: S-phase kinase-associated protein 2.

human cancers[21-25]. p27^{kip1} is a negative regulator of the cell cycle that plays an important role in tumor suppression. Loss of p27^{kip1} secondary to enhanced ubiquitin-mediated degradation results in uncontrolled proliferation and promotes tumor progression. In the present study, the positive immunoreactivity of Skp2 and Cks1 were noted in a significant proportion of patients with extrahepatic cholangiocarcinoma, and the positive immunoreactivity of Skp2 was a significant and independent prognostic factor in these patients. The expression of Skp2 was examined in a previous study by Sanada *et al*[27], in which they found a significant association between in-

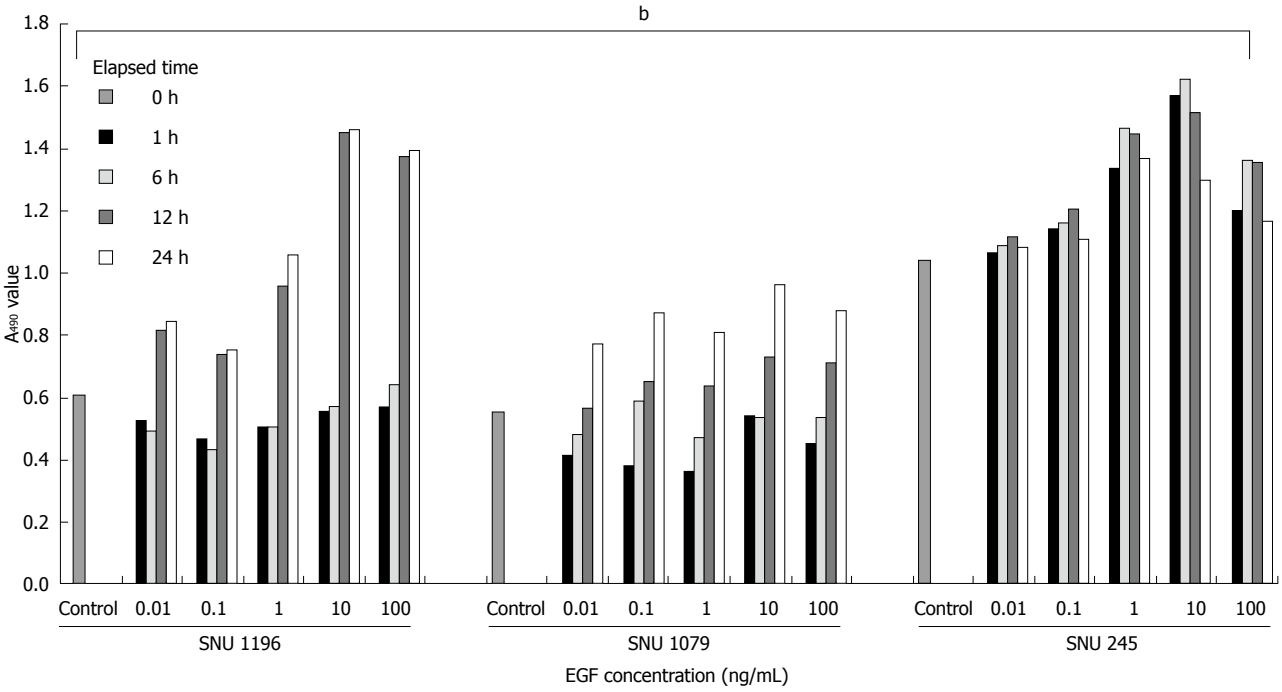


Figure 4 MTT assay after the addition of exogenous epidermal growth factor. Exogenous epidermal growth factor (EGF) (especially 0.1-10 ng/mL) significantly increased the proliferation indices of SNU-1196, SNU-1079, and SNU-245 cells. Statistical analyses were performed by one-way ANOVA. $^{b}P < 0.01$ *vs* control. Data are representative of triplicate experiments.

Table 2 Independent and significant prognostic factors for patients with extrahepatic cholangiocarcinoma

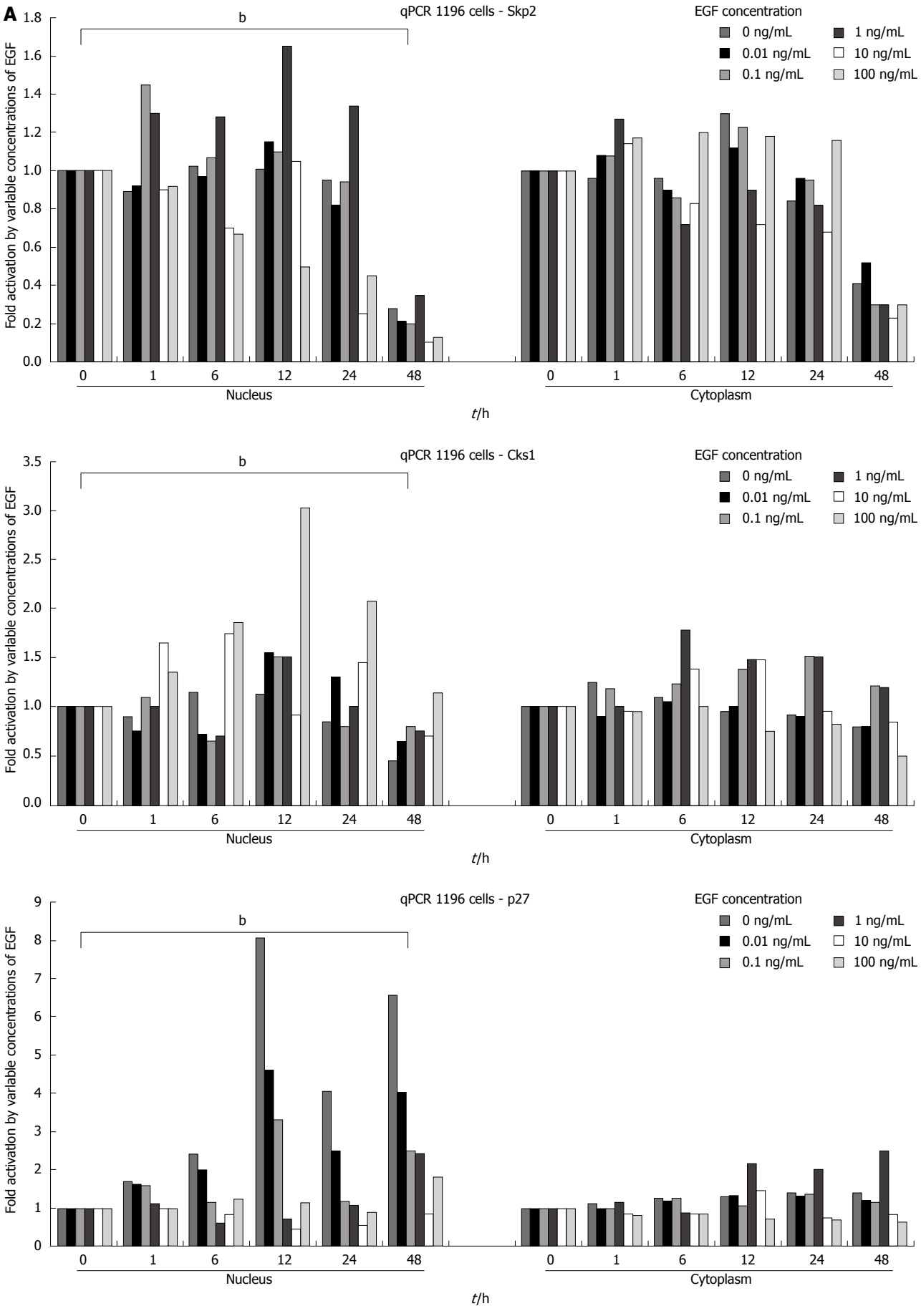
Parameters	Category	OR	95%CI	*P* value
Age	> 65 yr	2.707	1.341-5.464	< 0.01
AJCC stage	Ⅰ A			
	Ⅰ B	2.543	0.695-9.306	0.159
	Ⅱ A	0.801	0.185-3.477	0.767
	Ⅱ B	3.534	1.041-11.997	0.043
	Ⅲ	16.446	1.370-197.470	0.027
	Ⅳ	92.965	5.585-1547.531	< 0.010
Histologic type	Well-differentiated			
	Moderately-differentiated	6.041	2.146-17.006	< 0.010
	Poorly-differentiated	5.620	1.586-19.917	< 0.010
Immunostaining intensity of Skp2	0			
	1	1.308	0.571-2.994	0.526
	2	3.782	1.581-9.051	< 0.010
	3	2.312	1.127-6.941	0.048

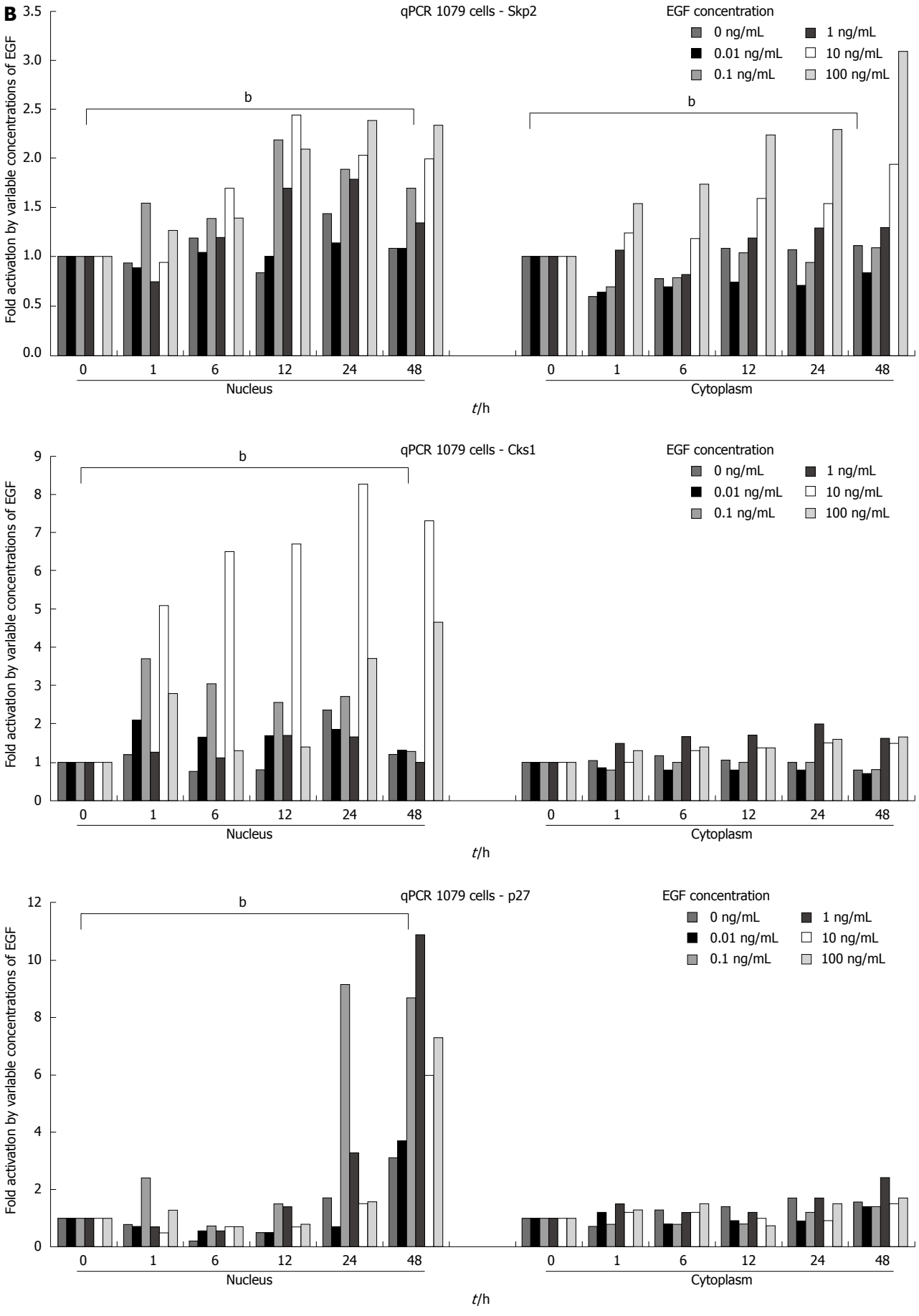
AJCC: American Joint Commission on Cancer; Skp2: S-phase kinase-associated protein 2.

creased levels of Skp2 protein and poor prognosis in patients with biliary tract carcinoma. However, in this study, the enrolled number of patients was relatively small (n = 33) and enrolled heterogeneous groups of patients, including extrahepatic bile duct and gallbladder cancers. In the current study, the staining intensity of Skp2 had significant linear correlations with those of Cks1 and nuclear p27^{kip1}. Notably, Ki-67 LI significantly increased as the staining intensity of Skp2 and Cks1 increased (P < 0.01, Figure 2). However, Ki-67 LI showed no significant correlations with the staining intensity of p27^{kip1}.

The lack of an inverse correlation between Skp2 and p27^{kip1} has been reported in several other types of solid tumors[28-30], as well as in biliary tract cancer[27]. These observations might be explained, at least partly, by additional oncogenic properties of Skp2 when it acts on target molecules other than p27^{kip1} and/or by additional molecular events involving p27^{kip1} proteolysis. Interestingly, the staining intensity of Ki67, a marker of cell proliferation, was significantly associated with that of nuclear p27^{kip1}. Our results indicate that the observations in which low levels of p27^{kip1} in aggressive human cancers may be caused by increased expression of Skp2 that targets p27^{kip1} for ubiquitin-mediated degradation did not apply to extrahepatic cholangiocarcinoma. The specific findings in need of alternative explanatory hypotheses in extrahepatic cholangiocarcinoma are the lack of an inverse correlation between p27^{kip1} and Skp2 levels and the apparent paradox of both increased expression of Skp2 and p27^{kip1}. Plausible explanations for these observations need further experiments. However, an independent mechanism of action for oncogenic Skp2 protein and tumor-suppressive p27^{kip1} in extrahepatic cholangiocarcinoma may be involved.

Conversely, it seems evident that oncoprotein Skp2 is an independent prognostic marker in patients with extrahepatic cholangiocarcinoma, like many other solid tumors[16-24]. It was repeatedly shown that Skp2 is an accurate and independent prognostic marker for disease-free and overall survival. Nevertheless, despite the increasing


A
qPCR 1196 cells - Skp2
EGF concentration
0 ng/mL
0.01 ng/mL
0.1 ng/mL
1 ng/mL
10 ng/mL
100 ng/mL
Fold activation by variable concentrations of EGF
b
Nucleus
Cytoplasm
t/h
qPCR 1196 cells - Cks1
qPCR 1196 cells - p27


B
qPCR 1079 cells - Skp2
EGF concentration
0 ng/mL
0.01 ng/mL
0.1 ng/mL
1 ng/mL
10 ng/mL
100 ng/mL
Fold activation by variable concentrations of EGF
b
Nucleus
Cytoplasm
t/h
qPCR 1079 cells - Cks1
qPCR 1079 cells - p27

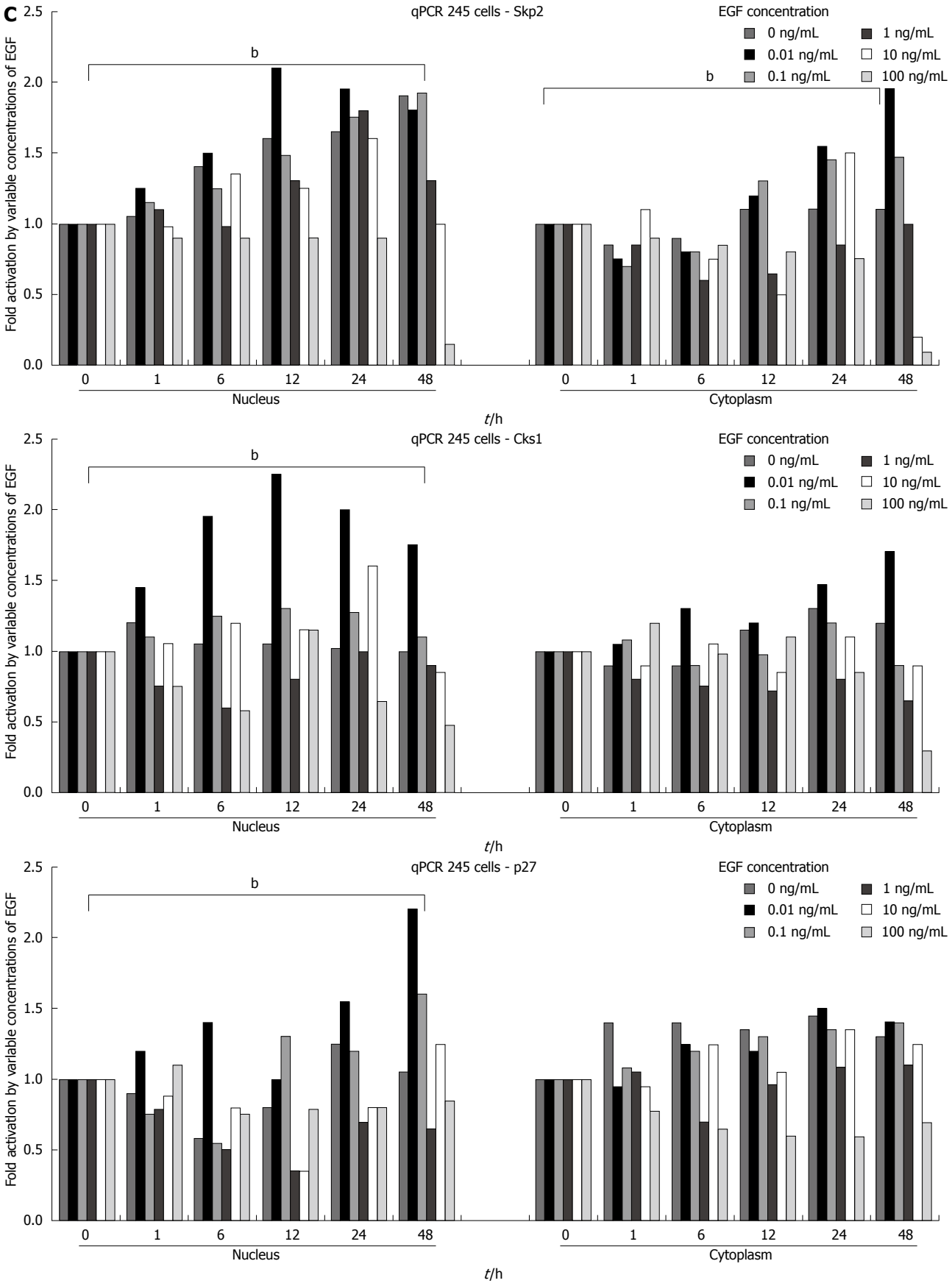
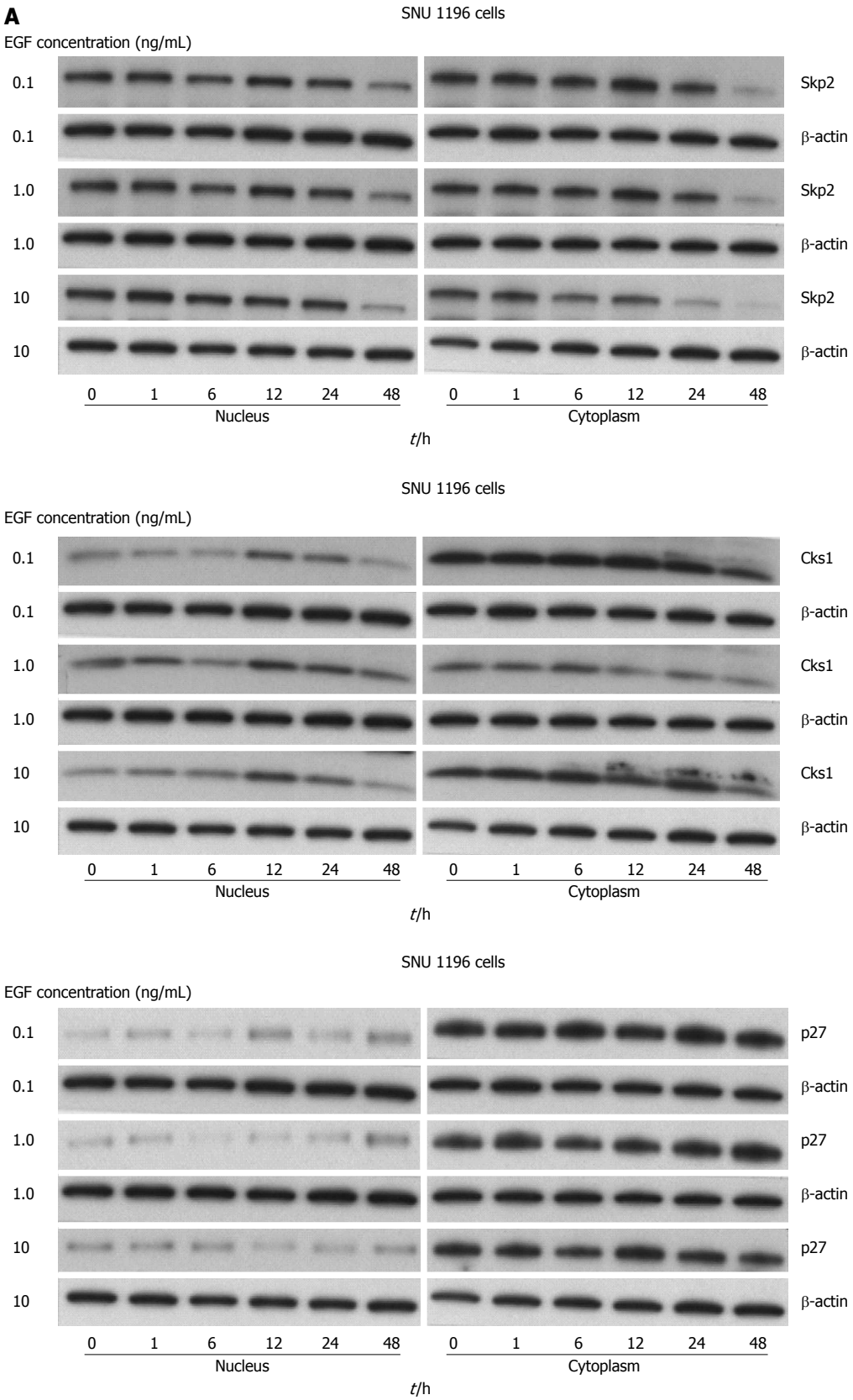
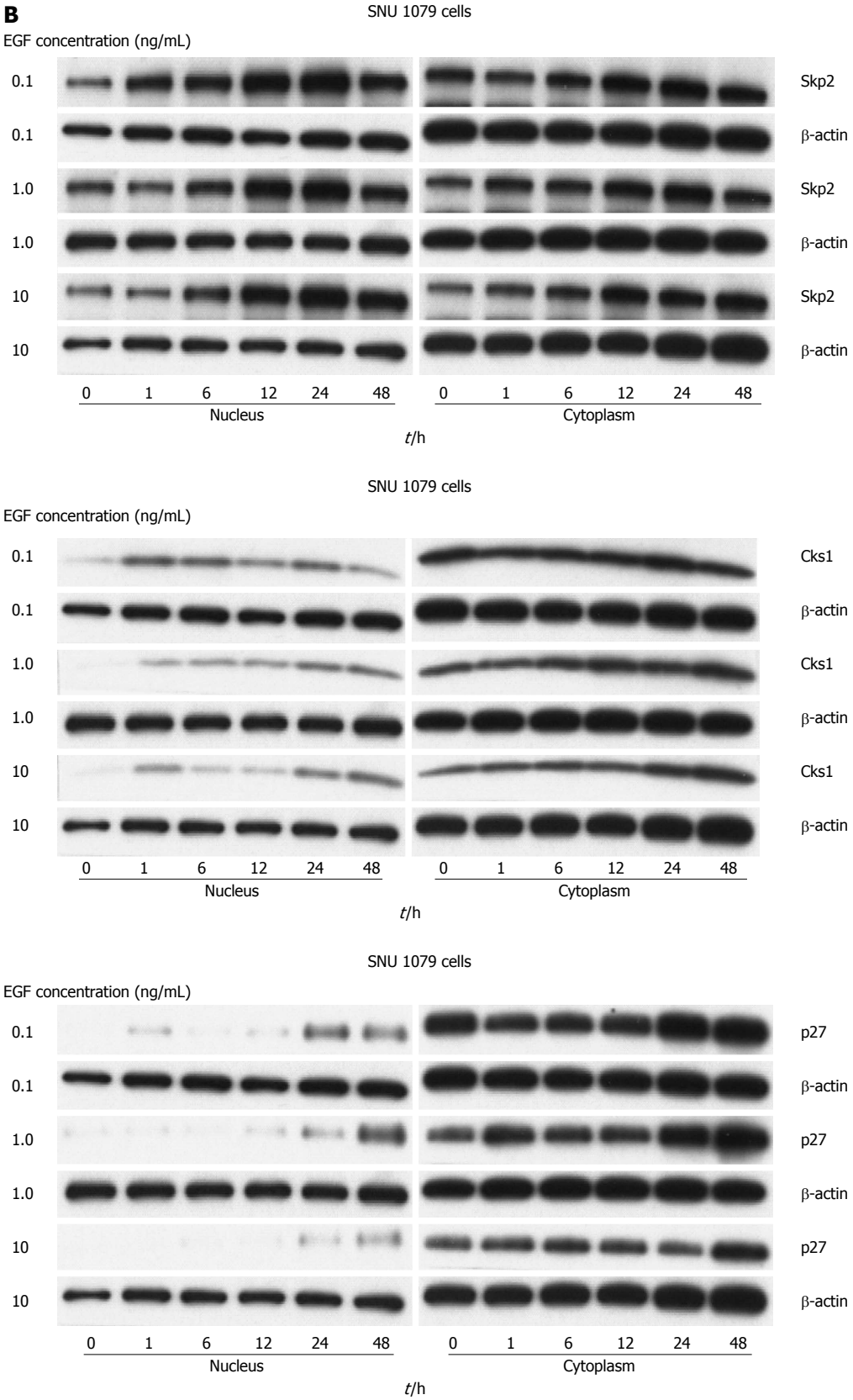


Figure 5 Results of quantitative polymerase chain reaction for S-phase kinase-associated protein 2, Cyclin-dependent kinases regulatory subunit 1, and p27kip1 after the addition of exogenous epidermal growth factor in (A) SNU-1196, (B) SNU-1079, and (C) SNU-245 cells. Data are representative of triplicate experiments. Statistical analyses were performed by one-way ANOVA. $^{b}P < 0.01$ *vs* control. EGF: Exogenous epidermal growth factor; Skp2: S-phase kinase-associated protein 2; Cks1: Cyclin-dependent kinases regulatory subunit 1.

A


SNU 1196 cells
EGF concentration (ng/mL)
0.1
0.1
1.0
1.0
10
10
Skp2
β-actin
Skp2
β-actin
Skp2
β-actin
0 1 6 12 24 48
Nucleus
0 1 6 12 24 48
Cytoplasm
t/h
SNU 1196 cells
EGF concentration (ng/mL)
0.1
0.1
1.0
1.0
10
10
Cks1
β-actin
Cks1
β-actin
Cks1
β-actin
0 1 6 12 24 48
Nucleus
0 1 6 12 24 48
Cytoplasm
t/h
SNU 1196 cells
EGF concentration (ng/mL)
0.1
0.1
1.0
1.0
10
10
p27
β-actin
p27
β-actin
p27
β-actin
0 1 6 12 24 48
Nucleus
0 1 6 12 24 48
Cytoplasm
t/h

B


SNU 1079 cells
EGF concentration (ng/mL)
0.1
0.1
1.0
1.0
10
10
Skp2
β-actin
Skp2
β-actin
Skp2
β-actin
0 1 6 12 24 48
Nucleus
0 1 6 12 24 48
Cytoplasm
t/h
SNU 1079 cells
EGF concentration (ng/mL)
0.1
0.1
1.0
1.0
10
10
Cks1
β-actin
Cks1
β-actin
Cks1
β-actin
0 1 6 12 24 48
Nucleus
0 1 6 12 24 48
Cytoplasm
t/h
SNU 1079 cells
EGF concentration (ng/mL)
0.1
0.1
1.0
1.0
10
10
p27
β-actin
p27
β-actin
p27
β-actin
0 1 6 12 24 48
Nucleus
0 1 6 12 24 48
Cytoplasm
t/h

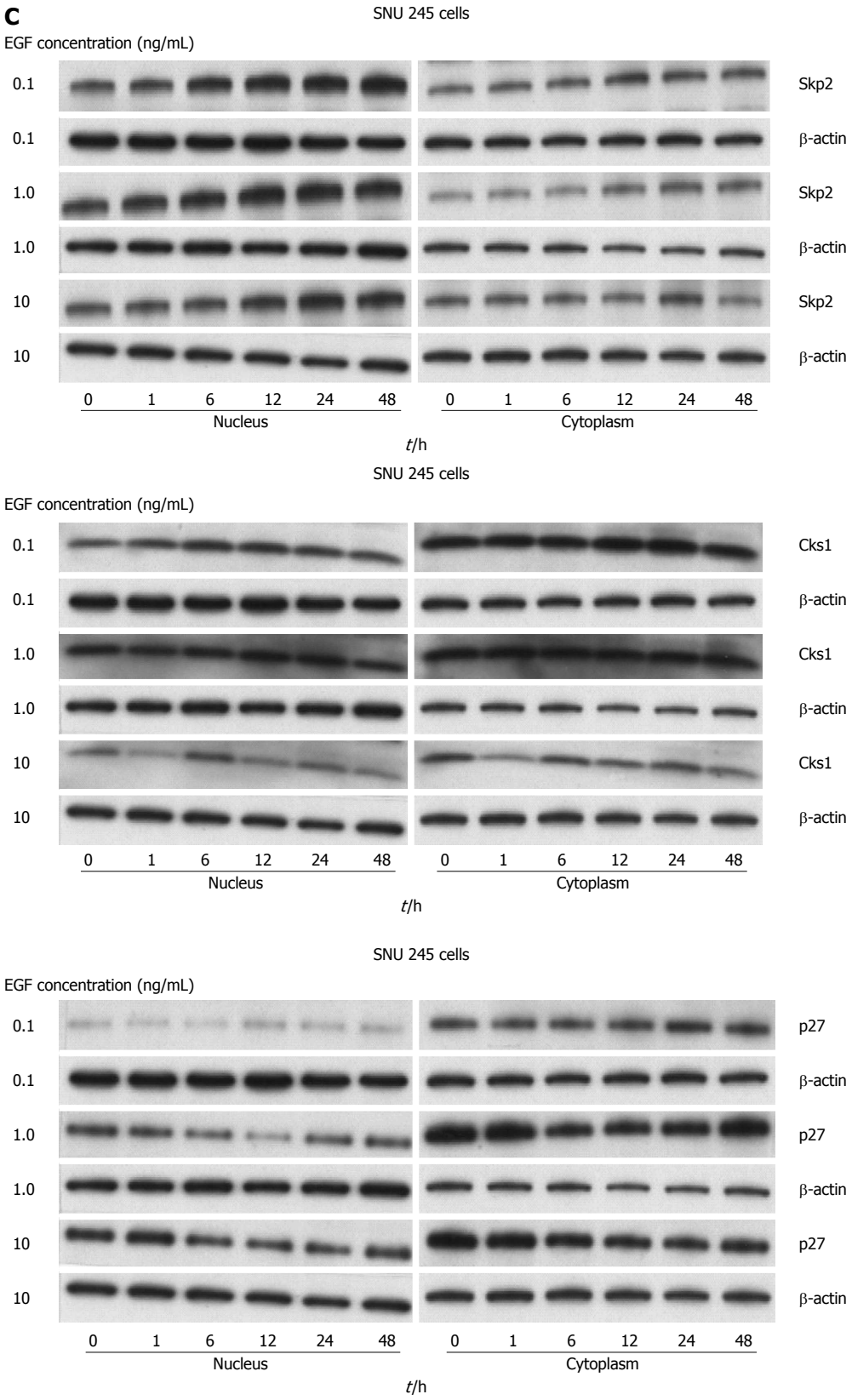
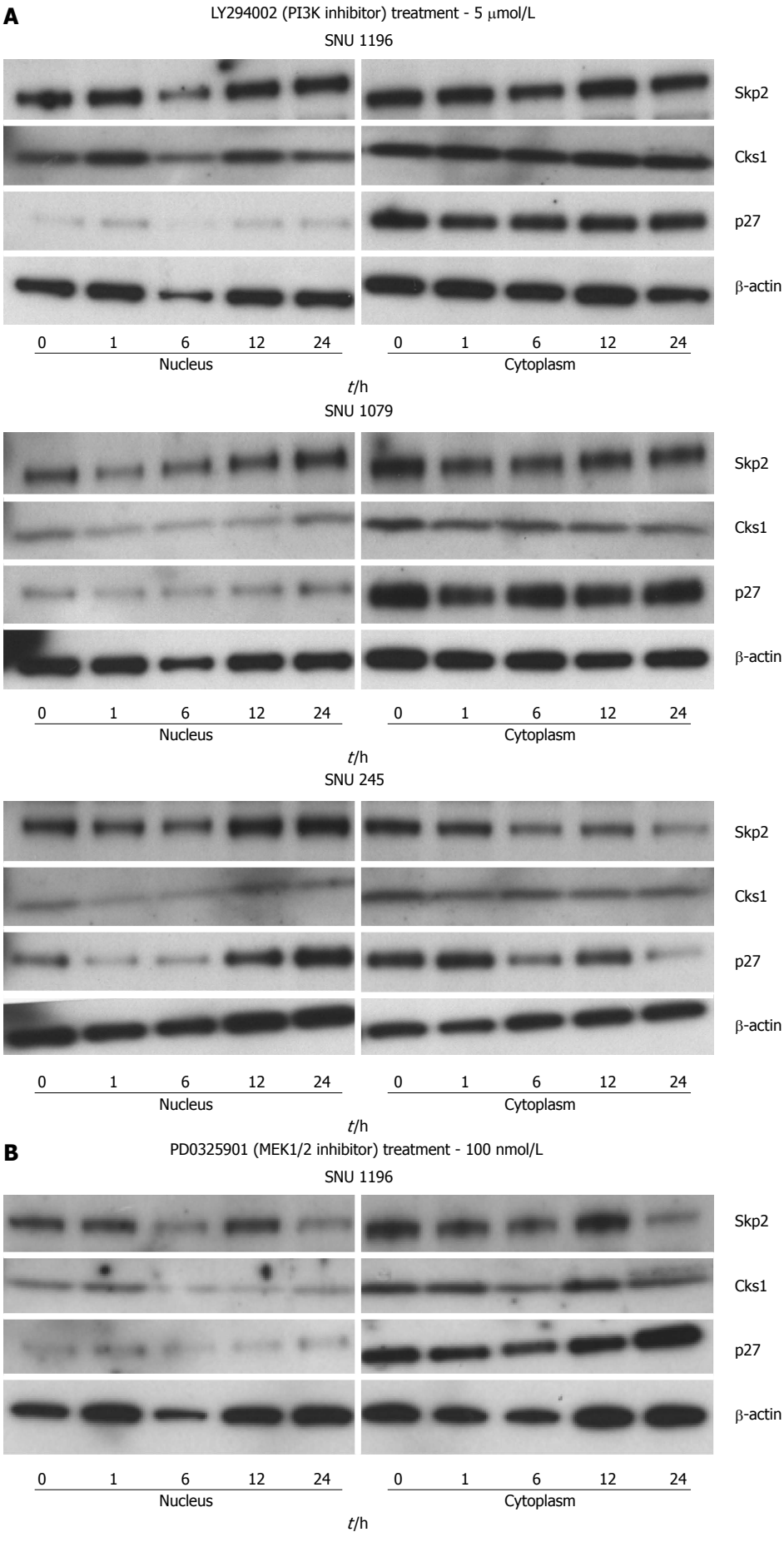


Figure 6 Results of western blotting for S-phase kinase-associated protein 2, Cyclin-dependent kinases regulatory subunit 1, and p27^{kip1} after the addition of exogenous epidermal growth factor in (A) SNU-1196, (B) SNU-1079, and (C) SNU-245 cells. Data are representative of triplicate experiments. Skp2: S-phase kinase-associated protein 2; Cks1: Cyclin-dependent kinases regulatory subunit 1.

evidence showing that Skp2 may more accurately stratify and subgroup patients at risk, there are no studies at present that have clinically implicated its application in the clinical decision process, such as adding adjuvant chemotherapy in patients with extrahepatic cholangiocarcinoma who underwent cure-intent surgical resection.


A
LY294002 (PI3K inhibitor) treatment - 5 μmol/L
SNU 1196
Skp2
Cks1
p27
β-actin
0 1 6 12 24
Nucleus
0 1 6 12 24
Cytoplasm
t/h
SNU 1079
Skp2
Cks1
p27
β-actin
0 1 6 12 24
Nucleus
0 1 6 12 24
Cytoplasm
t/h
SNU 245
Skp2
Cks1
p27
β-actin
0 1 6 12 24
Nucleus
0 1 6 12 24
Cytoplasm
t/h
B
PD0325901 (MEK1/2 inhibitor) treatment - 100 nmol/L
SNU 1196
Skp2
Cks1
p27
β-actin
0 1 6 12 24
Nucleus
0 1 6 12 24
Cytoplasm
t/h

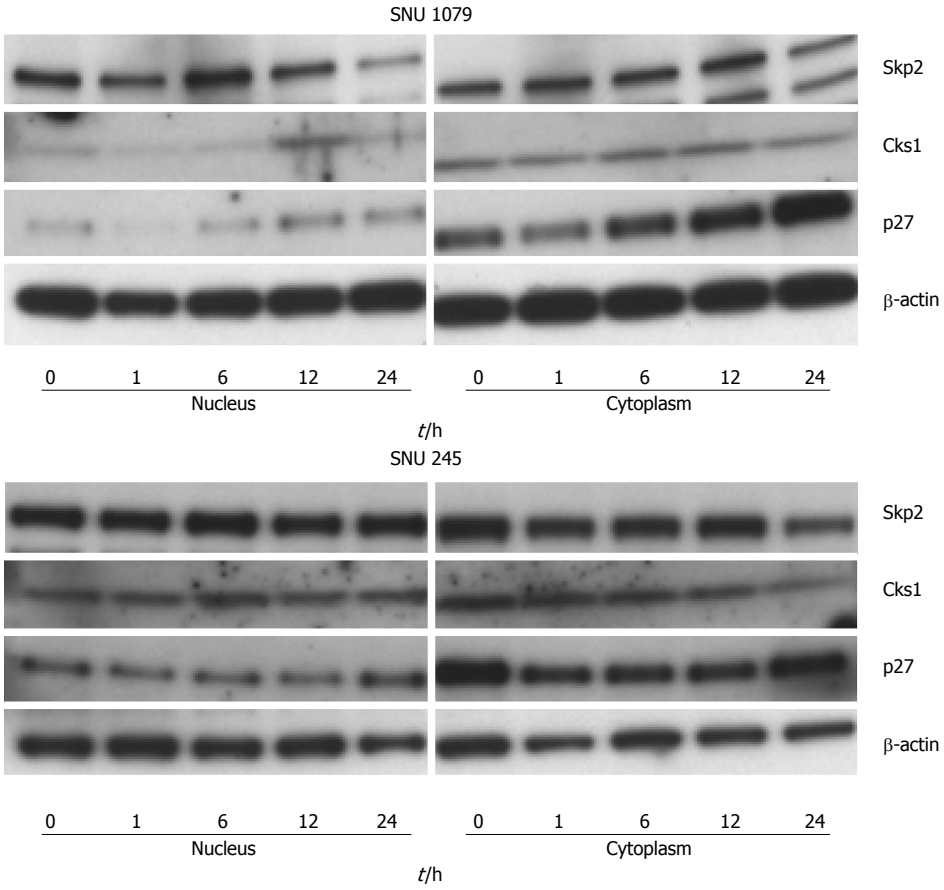


Figure 7 Effect of LY294002 (PI3K inhibitor, 5 μmol/L), and PD0325901 (MEK1/2 inhibitor, 100 nmol/L) on the protein levels of S-phase kinase-associated protein 2/Cyclin-dependent kinases regulatory subunit 1 and p27^{kip1} in SNU-1196, SNU-1079, and SNU-245 cells. Data are representative of triplicate experiments. Skp2: S-phase kinase-associated protein 2; Cks1: Cyclin-dependent kinases regulatory subunit 1.

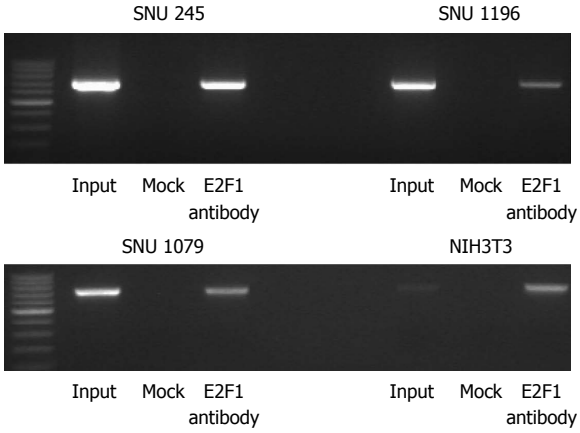


Figure 8 Binding of E2F1 transcription factor to the promoter region of *Skp2* gene. Chromatin immunoprecipitation assay shows the direct binding of E2F1 transcription factor to the promoter region of the *Skp2* gene after the addition of 10 ng/mL of EGF in SNU-245, SUN-1196, SUN-1079, and NIH3T3 cells.

The precise mechanisms that regulate Skp2 expression in cancer are not fully understood at present. Identification of the regulatory mechanism(s) leading to increased Skp2 expression may offer new insight into the control of tumor cell proliferation. A previous report[29] showed that *Skp2* gene amplification is likely to be associated with an advanced stage of tumor progression, while other control mechanisms not involving a gross genomic alteration are involved in the initial accumulation of Skp2 mRNA during oncogenesis. This implies a change in the rate of either transcription or mRNA degradation. Their analyses revealed no significant differences in the half-life of Skp2 mRNA between non-transformed and transformed human cell lines. Therefore, one plausible mechanism is a dysregulated transcription of Skp2 mRNA and a resultant increased level of Skp2 protein. The roles of EGF in human cholangiocarcinoma cells were reported to be sustained EGF receptor (EGFR) activation, extended -42/44 MAPK activation, and increased cell proliferation[31]. EGFR kinase inhibitors also effectively attenuated cellular growth of human cholangiocarcinoma cells. In this study, we show for the first time that transcription of Skp2 mRNA and translation of Skp2 protein were significantly increased after the exogenous addition of variable concentrations of EGF in human cholangiocarcinoma cell lines. A previous report[32] showed that Skp2 is a novel target of E2F regulation and that the *SKP2* gene contains a functional E2F response element. Moreover, an increase in DP-1, a coactivator of the E2F family and E2F3, was reported to be involved in the proliferation-enhancing effect of EGF[33]. In order to elucidate the transcriptional control of *Skp2* in human cholangiocarcinoma cells, we

performed ChIP assays after the addition of 10 ng/mL EGF, with the results showing that the E2F1 transcription factor directly binds to the promoter site of *Skp2* (Figure 5). E2F1 transcription factor is known to be a downstream target of variable growth factors, such as EGF and TGF-α. Disruption of the E2F/RB regulatory pathway is known to be a major contributor to increased E2F expression in many human tumors. Our finding that E2F1 that directly binds to the *SKP2* promoter offers a relevant mechanism for the upregulated Skp2 expression in human cholangiocarcinoma cells and may be served as possible therapeutic targets in this devastating malignancy.

The PI3K/AKT signaling pathway controls fundamental processes of cancer cell biology like proliferation and cell survival[34]. The PI3K/AKT pathway is activated in cholangiocarcinoma cells[35]. So far, the molecular mechanisms linking PI3K/AKT signaling to the cell cycle machinery in cholangiocarcinoma cells have not been investigated in detail. Using the PI3K inhibitor LY294002, we show that Skp2 is regulated by the PI3K/AKT pathway in cholangiocarcinoma cells. At the molecular level, the control of Skp2 protein expression is thought to be due to the regulation of E2F1 binding to the *Skp2* gene promoter.

In summary, higher immunostaining intensity of Skp2 was an independent prognostic factor for patients with extrahepatic cholangiocarcinoma. EGF upregulates the mRNA and protein levels of Skp2/Cks1 and p27^{kip1} *via* the PI3K/Akt pathway and direct binding of E2F1 transcription factor with the *Skp2* promoter.

COMMENTS

Background

Human extrahepatic cholangiocarcinoma (CC) is a highly malignant epithelial cancer of the biliary tract with high morbidity and mortality. Despite the current knowledge of etiology and pathology of CC, its cellular and molecular pathogenesis is still poorly understood. S-phase kinase associated protein 2 (Skp2) is the substrate-recognition subunit of the SCF^{skp2} E3 ligase complex. The important role of Skp2 in promoting entry into the S-phase, mainly by promoting p27^{kip1} destruction, was observed in cell-free systems, cell cultures, and animal models. In some cancers, including breast, colorectal, and gastric cancers, Skp2 and cyclin-dependent kinases regulatory subunit 1 (Cks1) expression were independent prognostic markers and provided additional prognostic information in these cancer patients. To our knowledge, there have been no reports concerning the expression status and the prognostic implication of Skp2 and Cks1 in extrahepatic cholangiocarcinoma.

Research frontiers

The research hotspot is exploring the cellular and molecular pathogenetic mechanisms of extrahepatic cholangiocarcinoma, which is highly malignant tumor with few or no palliative medical treatments available for unresectable patients.

Innovations and breakthroughs

The precise mechanisms that regulate Skp2 expression in cancer are not fully understood at present. Identification of the regulatory mechanism(s) leading to increased Skp2 expression may offer new insight into the control of tumor cell proliferation. The roles of exogenous epidermal growth factor (EGF) in human cholangiocarcinoma cells were reported to be sustained EGF receptor (EGFR) activation, extended -42/44 MAPK activation, and increased cell proliferation. EGFR kinase inhibitors also effectively attenuated cellular growth of human cholangiocarcinoma cells. In this study, the authors show for the first time that transcription of Skp2 mRNA and translation of Skp2 protein were significantly increased after the exogenous addition of variable concentrations of EGF in human cholangiocarcinoma cell lines. In order to elucidate the transcriptional control of *Skp2* in human cholangiocarcinoma cells, the authors performed chromatin immunoprecipitation assays after the addition of 10 ng/mL EGF, and the results showed that E2F1 transcription factor directly binds to the promoter site of *Skp2*. The finding that E2F1 directly binds to the *Skp2* promoter offers a relevant mechanism for the upregulated Skp2 expression in human cholangiocarcinoma cells, and may be served as a possible therapeutic target in this devastating malignancy.

Applications

The study results suggest that EGFR, the PI3K-Akt pathway, SKP2, and E2F1 can be candidate therapeutic targets in patients with extrahepatic cholangiocarcinoma

Terminology

Skp2: S-phase kinase associated protein 2, the substrate-recognition subunit of the SCF^{skp2} E3 ligase complex; Cks1: Cyclin kinase subunit 1, an additional protein that has a role in efficient interaction between the Skp2 ubiquitin ligase complex and its substrate p27^{kip1}; p27^{kip1}: Cyclin-dependent kinase inhibitor 1B, a protein which belongs to the *Cip/Kip* family of cyclin dependent kinase inhibitor proteins; PI3K: Phosphoinositide 3-kinases, a family of related intracellular signal transducer enzymes capable of phosphorylating the 3 position hydroxyl group of the inositol ring of phosphatidylinositol.

Peer review

This study investigated the role of the Skp2 signaling pathway in the pathogenesis of human extrahepatic cholangiocarcinoma. The paper is well written and results are interesting.

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ORIGINAL ARTICLE

Shunting branch of portal vein and stent position predict survival after transjugular intrahepatic portosystemic shunt

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Abstract

AIM: To evaluate the effect of the shunting branch of the portal vein (PV) (left or right) and the initial stent position (optimal or suboptimal) of a transjugular intrahepatic portosystemic shunt (TIPS).

METHODS: We retrospectively reviewed 307 consecutive cirrhotic patients who underwent TIPS placement for variceal bleeding from March 2001 to July 2010 at our center. The left PV was used in 221 patients and the right PV in the remaining 86 patients. And, 224 and 83 patients have optimal stent position and sub-optimal stent positions, respectively. The patients were followed until October 2011 or their death. Hepatic encephalopathy, shunt dysfunction, and survival were evaluated as outcomes. The difference between the groups was compared by Kaplan-Meier analysis. A Cox regression model was employed to evaluate the predictors.

RESULTS: Among the patients who underwent TIPS to the left PV, the risk of hepatic encephalopathy ($P = 0.002$) and mortality were lower ($P < 0.001$) compared to those to the right PV. Patients who underwent TIPS with optimal initial stent position had a higher primary patency ($P < 0.001$) and better survival ($P = 0.006$) than those with suboptimal initial stent position. The shunting branch of the portal vein and the initial stent position were independent predictors of hepatic encephalopathy and shunt dysfunction after TIPS, respectively. And, both were independent predictors of survival.

CONCLUSION: TIPS placed to the left portal vein with optimal stent position may reduce the risk of hepatic encephalopathy and improve the primary patency rates, thereby prolonging survival.

Key words: Transjugular intrahepatic portosystemic shunt; Cirrhosis; Variceal bleeding; Portal vein; Stent position

Core tip: This study reported the long-term follow-up results of a large cohort of cirrhotic patients who underwent transjugular intrahepatic portosystemic shunt

(TIPS) for variceal bleeding. The results demonstrated that the use of the left portal vein (PV) during the TIPS procedure could reduce post-TIPS hepatic encephalopathy risk and improve patient survival when compared to the use of the right PV, and that the deployment of a stent with optimal stent position could reduce the incidence of shunt dysfunction and benefit patient survival when compared to the deployment of a stent with suboptimal stent position.

Bai M, He CY, Qi XS, Yin ZX, Wang JH, Guo WG, Niu J, Xia JL, Zhang ZL, Larson AC, Wu KC, Fan DM, Han GH. Shunting branch of portal vein and stent position predict survival after transjugular intrahepatic portosystemic shunt. *World J Gastroenterol* 2014; 20(3): 774-785 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i3/774.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i3.774

INTRODUCTION

Transjugular intrahepatic portosystemic shunt (TIPS) placement is accepted worldwide as a means to decompress portal hypertension and to alleviate variceal hemorrhage[1-6]. However, this procedure has two major risks, which include hepatic encephalopathy (HE) (which is seen in somewhere between 21%-77% of patients per year)[1,2,7,8] and shunt dysfunction (which occurs in anywhere from 14%-82% of patients per year)[9-15].

Previous studies have reported that creation of a TIPS to the left portal vein (PV) instead of the right PV can decrease the risk of HE[16,17]. However, the sample sizes of these studies were relevantly small (the largest study included only 80 patients)[17]. One of these two studies evaluated the survival of patients who underwent left *vs* right PV TIPS and demonstrated that there was no difference in the survival between these two groups of patients[16].

In addition, several studies have implied that TIPS stents, when extended to the hepato-caval junction [rather than terminating in the hepatic vein (HV)], have a decreased incidence of shunt dysfunction[13,18,19]. However, these studies have not addressed the effect of stent position upon patient survival. Furthermore, to our knowledge, no study has evaluated the effect of the shunting branch of the PV and stent position concurrently.

This study aims to evaluate the effect of the shunting branch of the PV and the initial stent position on patient prognosis, particularly patient survival, in a large series of cirrhotic patients who underwent TIPS for variceal bleeding.

MATERIALS AND METHODS

Study population

Between March 2001 and July 2010, all consecutive patients with cirrhosis who underwent a TIPS procedure for an indication of variceal bleeding at our center were retrospectively analyzed in the present study (both in the elective and emergency settings). The exclusion criteria are as follows: (1) PV thrombosis; (2) hepatocellular carcinoma; (3) other malignant diseases; (4) sepsis; (5) renal failure (serum creatinine > 265 μmol/L); (6) heart failure; and (7) age < 18 years. Local ethical committee approved the study protocol. Written informed consent for TIPS procedure was obtained from every patient.

Definitions and endpoints

Liver cirrhosis was diagnosed according to a history of liver disease, decreased liver function, portal hypertension and characteristic imaging features suggesting cirrhosis. A biopsy was performed when hepatocellular carcinoma was suspected. The diagnosis of PV thrombosis was established mainly based on color Doppler ultrasound (CDUS) and computed tomography as our previous description[20]. The definition of technical success was successful creation of a TIPS between the HV and PV and reduction of the portosystemic gradient (PSG) to < 12 mmHg or by > 25%[21].

HE was diagnosed and classified according to the West Haven criteria[22]. Grade Ⅲ and Ⅳ were considered to represent severe HE. Recurrent (at least three episodes of HE in the last 3 mo) and/or persistent HE (continuously detectable altered mental state with further episodic deteriorations) despite protein restriction and active medical treatment were considered to be refractory HE[23].

Shunt dysfunction was suggested when any one of the following events was observed: (1) variceal bleeding; (2) occurrence of severe ascites; or (3) a maximum flow velocity < 50 cm/s or the absence of flow within the shunt as demonstrated by CDUS. Suspected shunt dysfunction was confirmed by portography and a pressure measurement that showed a PSG > 15 mmHg[20]. The duration of time from the TIPS procedure to the first shunt dysfunction was defined as the primary patency.

Optimal initial stent position (O-SP) (Figure 1) was defined as a stent position that satisfied the following two criteria: (1) the cephalic end of the stent extended to the hepato-caval junction[18]; and (2) the caudal end of the stent was parallel to the vascular wall of the PV (the angle between the tangent line of the caudal end of the stent and the vascular wall of the PV was less than 20°). Otherwise, the stent position was considered to be suboptimal. We reviewed the stent position by anteroposterior and lateral imaging. If the position was identified as suboptimal by either imaging view, the stent was diagnosed as a suboptimal initial stent position (sub-O-SP). The sub-O-SP diagnosis comprised of the following three categories: (1) suboptimal in the HV; (2) suboptimal in the PV; and (3) suboptimal in both the HV and PV. Based on the predefined criteria, the stent position of each patient was classified by two interventional radiologists with ten (C. H) or nine (W. G) years of experience, who were blinded to each other's classifications and to the patients' outcomes.

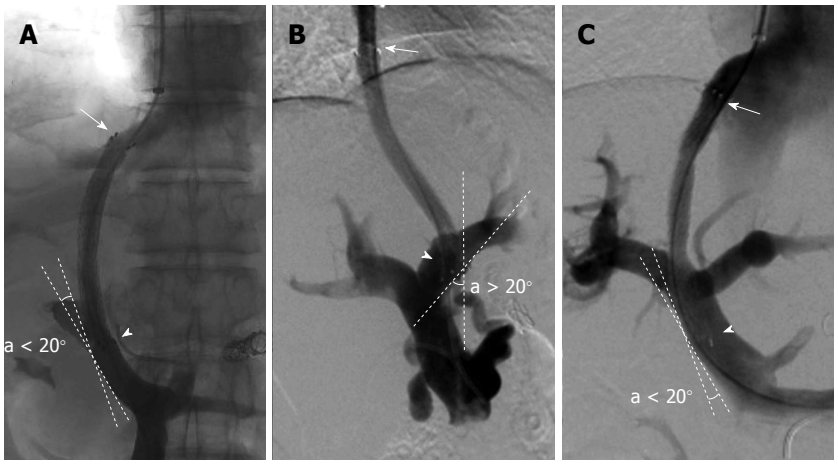


Figure 1 Stent position classifications. The initial stent position was classified according to the angiography imaging as follows: A: Suboptimal in the hepatic vein (HV) (arrow) and optimal in the portal vein (PV) (arrow head); B: Optimal in the HV (arrow) and suboptimal in the PV (arrow head); C: Optimal in the HV (arrow) and optimal in the PV (arrow head).

TIPS procedure

The technique for creating a TIPS has been described previously[13,24,25]. After indirect portography (mesenteric artery angiography) was performed, the HV (commonly, the right HV) was reached using a TIPS set (RUPS-100, Cook, Cook Inc., Bloomington, IL, United States), and the PV was punctured under the guiding of digital subtraction angiography. The following four steps were employed to puncture the PV: (1) the anatomical position between HV and PV was estimated by CT imaging and indirect portography; (2) the metal cannula of the RUPS-100 was bended to an appropriate angle (usually 30^{o}-70^{o} for the left and 20^{o}-50^{o} for the right PV, respectively) according to the estimated anatomical position; (3) the RUPS-100 was introduced into the right HV; and (4) the end of the RUPS-100 was turned to a point at the left/right PV and then the targeted PV could be successfully punctured. For an experienced expert, either the left or right PV can be selected and successfully punctured, which have been proved in previous studies[16,17]. Which PV branch was punctured was determined by the interventional radiologists and recorded at the time of the TIPS procedure. A 10-mm stent was used for TIPS creation before October 2006 and an 8-mm stent (BARD, Luminexx, Voisins le Bretonneux, France) was used thereafter to avoid excessive portosystemic shunting. Furthermore, additional dilations were performed whenever the PSG was > 12 mmHg or the reduction in the PSG was < 25%[21]. Markedly enlarged gastroesophageal collateral vessels observed during the TIPS procedure were embolized with coils (Cook Incorporated, 750 Daniels Way Bloomington, IN). For the patients included in this study, covered stents could not be employed because the State Food and Drug Administration had not approved these stents at the time of the TIPS procedures.

After the TIPS procedure, intravenous heparin (8000-12000 u/d) was given for 5-7 d and then warfarin for 6 mo and lifelong aspirin were prescribed at dosages to achieve an international normalized ratio (INR) of up to two times the upper limit of normal to prevent shunt dysfunction[20]. The antithrombotic therapy strategy was made according to the practice guidelines of American College of Chest Physicians[26]. A TIPS revision was planned whenever shunt dysfunction was recognized.

Follow-up

The patients were followed until October 2011 or their death. Variceal bleeding, ascites, HE, and survival were assessed at one, three, six and 12 mo and then yearly. Blood tests, coagulation function tests (prothrombin time, INR) and CDUS (diameter, flow velocity and direction of flow in the PV and shunt), if possible, were obtained at the follow-up time points and any time when symptoms recurred (hematemesis, melena or large volume ascites).

Statistical methods

Numerical variables are expressed as mean value ± standard deviation. Normal continuous variables were compared using the Student's t test. Non-normal continuous variables were compared using the Mann-Whitney rank-sum test. Nominal variables are expressed as frequencies and compared using the χ^2 test. Accumulated proportions were assessed using Kaplan-Meier curves and compared using the log-rank test. The following items were included in univariate analyses: age, gender, etiology, procedure type (elective/emergency), ascites, previous HE, splenectomy, platelet count, serum albumin, bilirubin, creatinine, sodium concentrations, INR, Child-Pugh score, MELD score, PSG, stent diameter, shunting branch of the PV, initial stent position, HE within six months, shunt dysfunction within six months, and TIPS date. A Cox proportional regression hazards model was used to assess the prognostic value of the significant variables found in the univariate analyses. Patients who were lost to follow-up or underwent liver transplantation were censored at the last follow-up date and the date of transplantation, respectively. All of the statistical analyses

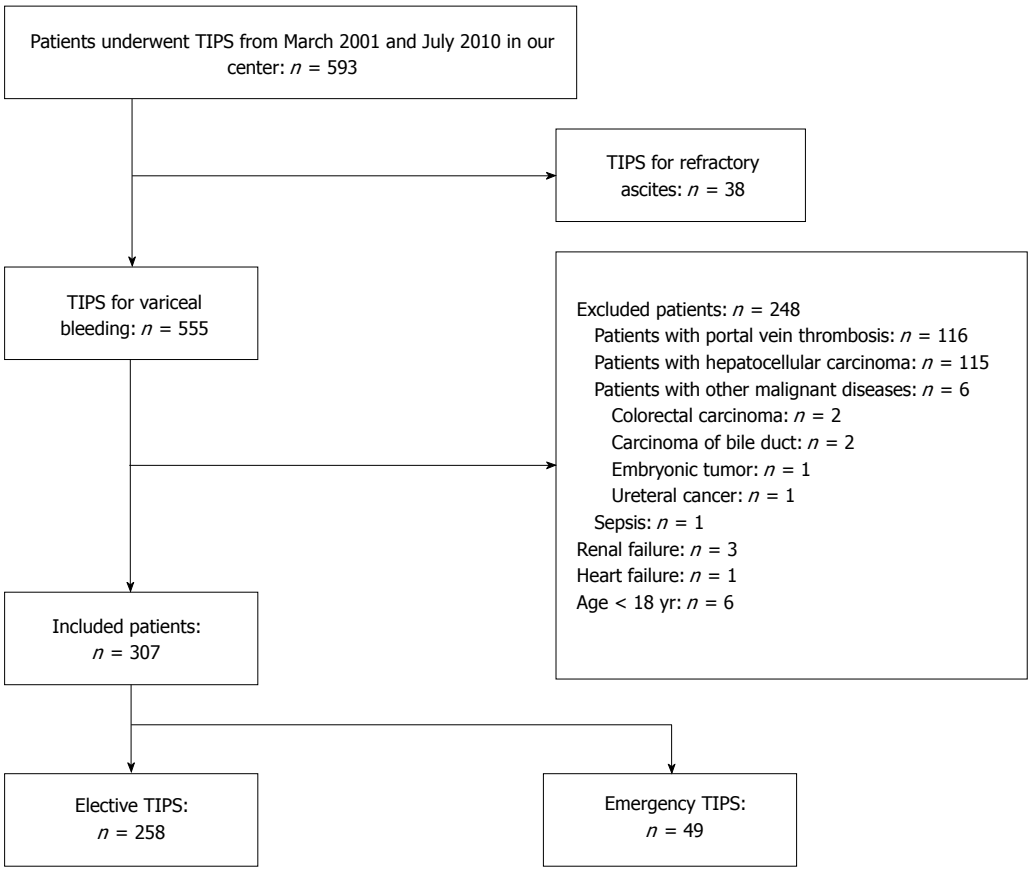


Figure 2 Selection flowchart for the consecutive patients who underwent transjugular intrahepatic portosystemic shunt between March 2001 and July 2010. TIPS: Transjugular intrahepatic portosystemic shunt.

were performed with SPSS 17.0 (SPSS, Chicago, IL), and a two-tailed P value < 0.05 was considered statistically significant.

RESULTS

Patient characteristics

Figure 2 presents the patient selection flowchart. The median follow-up time was 2.5 years (range, 0.1-10.9 years). Patient demographics of our cohort of 307 patients prior to TIPS creation are presented in Table 1. Of these patients, 258 underwent TIPS creation in elective settings and 49 in emergency settings. TIPS creation was technically successful in all of the included patients. The left PV was used in 221 patients and the right PV in the remaining 86 patients. For these two groups of patients, no significant difference was observed in the baseline clinical and laboratory characteristics (Table 1). In three of the included patients, intra-abdominal bleeding caused by an extrahepatic PV puncture was successfully treated by rapid decompression of the portal system with stent deployment. Other procedure-related complications included one patient with a bile duct injury and one patient with a liver capsule hematoma, both of whom are alive at the time of follow-up for this study.

Classification of the stent position

The position of the stent was classified as O-SP in 224 patients, sub-O-SP in 83 patients, including sub-O-SP in the HV in 63 patients, PV in 17 patients and both HV and PV in the remaining three patients. Table 1 demonstrates that patients with O-SP and those with sub-O-SP also had comparable baseline characteristics. A high inter-observer agreement was demonstrated between the two interventional radiologists who classified the initial stent position (κ = 0.98, P < 0.001). Disagreements for the three patients in whom a consensus was not reached were resolved through discussion.

HE

In total, 209 episodes of overt HE were observed in 128 patients. The majority (n = 71) of the first episodes of HE were grade Ⅱ. During the follow-up, 72 patients had one episodes of HE, and the rest had two or more episodes HE. HE were successfully controlled by medical treatments in all patients except the three who showed refractory HE and required a reduction of the stent size three, 10, and 17 mo after TIPS implantation. The proportions of patients remaining free of HE were 74.5% in three months, 68.1% in one year, and 56.8% in three years.

The proportion of patients free of HE after one and three years were 54.3% and 44.0% for the patients with a right PV TIPS *vs* 73.4% and 61.3% for the patients with a left PV TIPS (Figure 3A, log-rank test: P = 0.002). In the 8-mm stent (Figure 3C, log-rank test: P = 0.030) and 10-mm stent subgroups (Figure 3E, log-rank test: P =

Table 1 Characteristics of patients before transjugular intrahepatic portosystemic shunt

Characteristic	Total	Shunting branch of the PV		Initial stent position	
	(*n* = 307)	Left (*n* = 221)	Right (*n* = 86)	Optimal (*n* = 224)	Sub-optimal (*n* = 83)
Age (yr)	50.7 ± 12.8	50.0 ± 12.9	52.5 ± 12.3	50.8 ± 12.6	50.4 ± 13.3
Gender (male/female)	209/98	151/70	58/28	147/77	62/21
TIPS procedure (elective/emergency)	258/49	190/31	68/18	190/34	68/15
Etiology (viral/not viral[1])	275/32	199/22	76/10	197/27	78/5
Ascites (no/yes)	94/213	74/147	20/66	69/155	25/58
Previous HE (no/yes)	298/9	214/7	84/2	218/6	80/3
Previous splenectomy (no/yes)	252/55	179/42	73/13	184/40	68/15
Platelet count (10^9/L)	82.3 ± 65.4	82.3 ± 68.9	82.6 ± 55.8	85.5 ± 68.4	73.9 ± 56.2
Albumin (g/L)	33.3 ± 5.2	33.4 ± 5.0	32.9 ± 5.9	33.1 ± 5.2	33.8 ± 5.2
Total bilirubin (μmol/L)	24.7 ± 19.2	24.3 ± 18.1	25.7 ± 21.7	24.0 ± 17.8	26.4 ± 22.4
INR	1.3 ± 0.3	1.3 ± 0.3	1.3 ± 0.3	1.3 ± 0.3	1.3 ± 0.3
Serum creatinine (μmol/L)	82.4 ± 21.8	82.3 ± 21.9	82.6 ± 21.6	81.0 ± 21.2	86.2 ± 22.9
Sodium (mmol/L)	138.9 ± 4.7	139.0 ± 4.3	138.4 ± 5.6	138.6 ± 5.0	139.6 ± 3.6
Child-Pugh score	7.1 ± 1.6	7.1 ± 1.6	7.3 ± 1.7	7.1 ± 1.7	7.1 ± 1.5
Child-Pugh classification (A/B/C)	126/157/24	94/110/17	32/47/7	93/111/20	33/46/4
MELD score	11.1 ± 3.3	11.1 ± 3.3	11.1 ± 3.5	11.2 ± 3.5	10.8 ± 2.7
Pre-TIPS PSG (mmHg)	22.3 ± 4.5	22.5 ± 4.3	21.7 ± 4.9	22.4 ± 4.6	22.0 ± 4.2
Reduction ratio of PSG (%)	45.8 ± 17.4	46.3 ± 17.4	44.5 ± 17.3	45.7 ± 17.4	45.9 ± 17.5
Stent diameter (8-/10-mm)	206/101	150/71	56/30	150/74	56/27
Shunting branch of the PV (right/left)	86/221	-	-	57/164	26/60
Initial stent position (optimal/suboptimal)	224/83	164/57	60/26	-	-
TIPS date[2] (before 2006/after 2006)	118/189	75/146	43/43[3]	83/141	35/48
Lost to follow-up	19	12	7	15	4

[1]Cirrhosis caused by autoimmune diseases (*n* = 11), cirrhosis caused by medications (*n* = 4), schistosome (*n* = 1) or nonalcoholic steatohepatitis (*n* = 2); [2]The date of TIPS procedure; [3]The comparison between groups was significantly different. HE: Hepatic encephalopathy; PSG: Portosystemic pressure gradient; INR: International normalized ratio; MELD: Model for end-stage liver diseases; PV: Portal vein.

0.035), the patients with a left PV TIPS had a lower rate of post-procedure HE. Severe HE was observed in 21 (9.5%) patients in the left PV group and in 13 (15.1%) patients in the right PV group ($P = 0.159$). Of the three patients who presented refractory HE, two underwent a left and one underwent a right PV TIPS.

After univariate analysis (Table 2) and multivariate analysis, age ($P = 0.001$), INR ($P = 0.014$), Child-Pugh score ($P = 0.004$), MELD score ($P = 0.021$), reduction ratio in PSG ($P = 0.009$) and shunting branch of PV ($P = 0.014$) were identified as independent predictors of HE (Table 3).

Shunt dysfunction

During the follow-up, 118 patients had at least one episode of shunt dysfunction. Of these 118 patients, 80 underwent 108 TIPS revisions, 25 died due to the first post-TIPS variceal bleeding, five underwent endoscopic therapies and two underwent surgical therapies for variceal rebleeding. Six patients required large volume paracentesis due to severe ascites. The primary patency rates of our cohort after one, three, and five years were 79.9%, 58.7%, and 46.0%, respectively.

The 1-, 3- and 5-year primary patency rates were 83.3%, 66.7% and 53.5% for patients with O-SP and 70.9%, 38.2% and 25.8% for patients with sub-O-SP, respectively (Figure 4A, log-rank test: $P < 0.001$). Moreover, patients with each of the three types of sub-O-SP had a significantly higher risk of shunt dysfunction than patients with O-SP (Figure 5). In the 8-mm stent (Figure 4C, log-rank test: $P = 0.019$) and 10-mm stent subgroups (Figure 4E, log-rank test: $P < 0.001$), the primary patency rates in the patients with O-SP were significantly higher compared to those with sub-O-SP.

Among patients with shunt dysfunction and portography, those with sub-O-SP in the HV usually had stenosis or occlusion in the HV (86.2%), and those with sub-O-SP in the PV had stenosis or occlusion in the PV (83.3%). Patients with O-SP experienced stenosis or occlusion in the PV at a rate of 33.3% and in the HV at a rate of 33.3%. The differences among the three groups and between any two of these three groups were statistically significant (Figure 6).

Three variables were evaluated as potential risk factors for shunt dysfunction in univariate analyses (Table 2). The multivariate analysis revealed that previous splenectomy ($P = 0.008$) and initial stent position ($P < 0.001$) were independent predictors of primary patency.

Survival

Among the 110 deaths, underlying etiologies included liver failure in 56, variceal rebleeding in 42, hepatocellular carcinoma in four, and other causes in eight (which included cerebral hemorrhage, heart failure, myocardial infarction, car accident, encephalitis, diabetes, esophageal cancer and pancreatic cancer). The overt survival rates for the included patients were 89.0% at one year, 71.4% at three years, and 51.0% at five years with a median survival time of 5.6 years (95%CI: 4.3-6.9).

The median survival time was 8.4 years (95%CI:

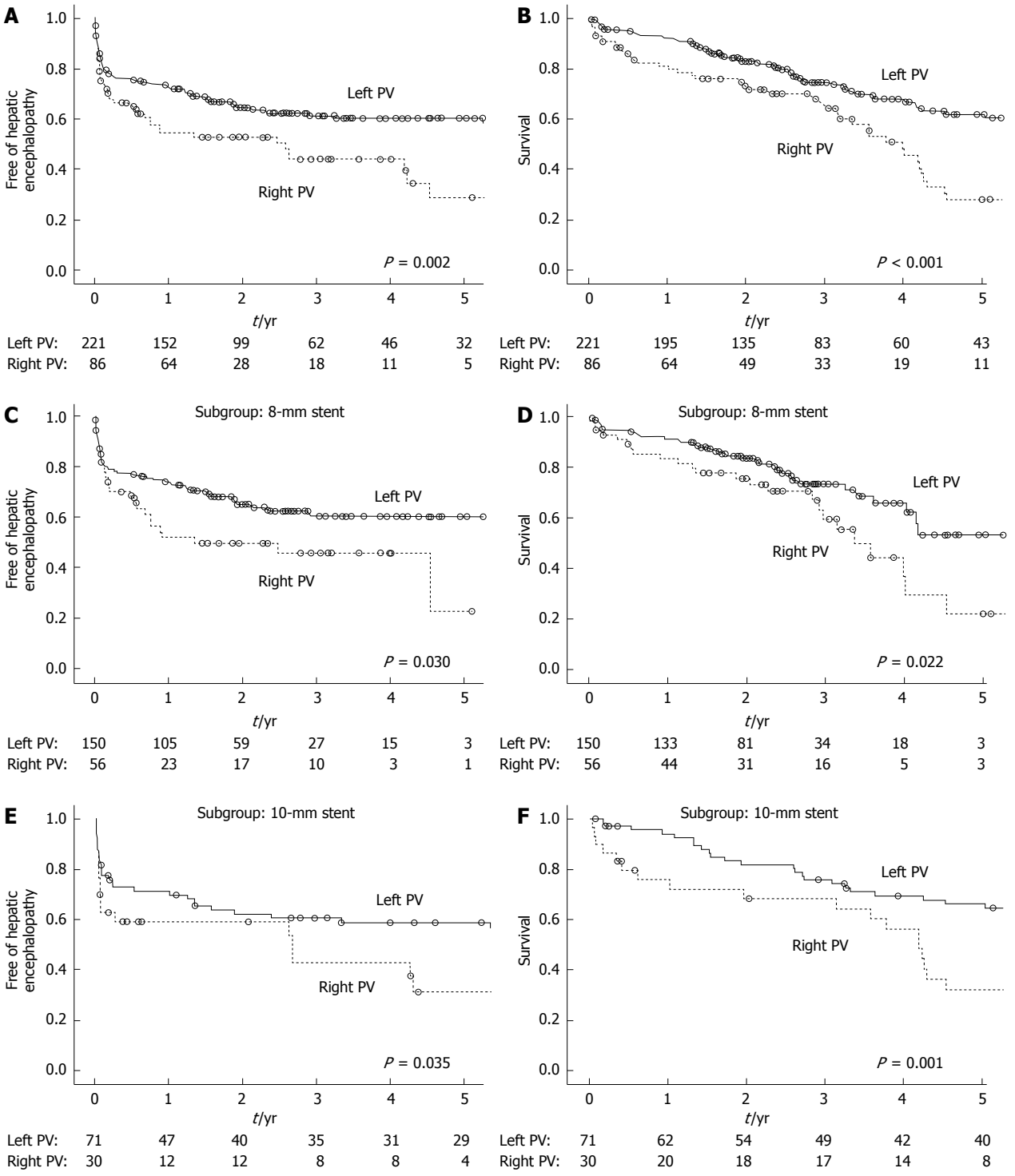


Figure 3 Hepatic encephalopathy results from the Kaplan-Meier analyses. Comparison of hepatic encephalopathy between the patients with a transjugular intrahepatic portosystemic shunt (TIPS) to the left portal vein (PV) and those with a TIPS to the right PV in all patients (A), an 8-mm stent subgroup (C) and a 10-mm stent subgroup (E). Comparisons of survival between the patients with a TIPS to the left PV and those with a TIPS to the right PV in all patients (B), an 8-mm stent subgroup (D) and a 10-mm stent subgroup (F).

5.2-11.5) for patients with a left PV TIPS and 3.9 years (95%CI: 3.3-4.7) for patients with a right PV TIPS (Figure 3B: log-rank test: $P < 0.001$). The patients with a left PV TIPS had a significantly higher survival rate compared to those with a right PV TIPS in both the 8-mm (Figure 3D, log-rank test: $P = 0.022$) and 10-mm stent subgroups (Figure 3F, log-rank test: $P = 0.001$).

For the patients with O-SP and those with sub-O-SP, the survival rates after one, three and five years were 90.4%, 75.8% and 56.6%, and 85.3%, 60.7% and 38.9%, respectively (Figure 4B: log-rank test: $P = 0.006$). In the 8-mm stent subgroup, the survival rate for the patients with O-SP was significantly higher compared to that for the patients with sub-O-SP (Figure 4D, log-rank test: $P =$

Table 2 Results from the univariate analyses

Variable	Hepatic encephalopathy			Shunt dysfunction			Survival		
	HR	95%CI	*P* value	HR	95%CI	*P* value	HR	95%CI	*P* value
Age (yr)	1.03	1.01-1.04	< 0.001	0.99	0.97-1.01	NS	1.01	0.99-1.03	NS
Gender (male/female)	1.04	0.72-1.50	NS	1.62	1.07-2.45	0.023	1.41	0.92-2.16	NS
TIPS procedure (elective/emergency)	1.37	0.88-2.13	NS	0.66	0.38-1.14	NS	1.57	1.02-2.41	0.041
Ascites (yes/no)	1.55	1.04-2.30	0.031	0.90	0.62-1.33	NS	2.22	1.38-3.57	0.001
Previous splenectomy (yes/no)	0.97	0.61-1.54	NS	1.65	1.09-2.50	0.018	0.61	0.36-1.02	NS
Platelet count (10^9/L)	1.00	0.99-1.01	NS	1.00	0.99-1.01	NS	0.99	0.99-1.00	0.021
Albumin (g/L)	0.96	0.93-0.99	0.022	0.98	0.95-1.02	NS	0.96	0.92-0.99	0.035
Total bilirubin (μmol/L)	1.00	0.99-1.01	NS	1.00	0.99-1.01	NS	1.01	1.00-1.02	0.002
INR	2.09	1.20-3.64	0.009	0.82	0.41-1.63	NS	3.12	1.86-5.23	< 0.001
Child-Pugh score	1.17	1.06-1.29	0.003	1.01	0.90-1.13	NS	1.29	1.15-1.44	< 0.001
MELD score	1.05	1.01-1.11	0.032	0.97	0.91-1.03	NS	1.09	1.04-1.14	< 0.001
Reduction ratio of PSG (%)	1.02	1.01-1.03	0.004	0.99	0.98-1.01	NS	1.01	0.99-1.02	NS
Shunting branch of the PV (right /left)	1.75	1.22-2.52	0.003	0.81	0.53-1.23	NS	2.10	1.43-3.07	< 0.001
Initial stent position (sub-optimal/optimal)	0.75	0.49-1.15	NS	2.24	1.54-3.25	< 0.001	1.88	1.44-2.47	< 0.001
HE within 6 mo (yes/no)	-	-	-	-	-	-	1.60	1.07-2.38	0.021
Shunt dysfunction within 6 mo (yes/no)	-	-	-	-	-	-	1.87	1.15-3.04	0.011

HE: Hepatic encephalopathy; PSG: Portosystemic pressure gradient; INR: International normalized ratio; MELD: Model for end-stage liver diseases; PV: Portal vein; NS: Non-significant; TIPS: Transjugular intrahepatic portosystemic shunt.

Table 3 Risk factors from the multivariate analyses

Variable	Multivariate analysis		
	HR	95%CI	*P* value
Hepatic encephalopathy			
Age	1.02	1.01-1.04	0.001
INR	2.01	1.15-3.50	0.014
Child-Pugh score	1.17	1.05-1.31	0.004
MELD	1.06	1.01-1.11	0.021
Shunting branch of the PV (right/left)	1.59	1.10-2.30	0.014
Reduction ratio of PSG (%)	1.01	1.00-1.03	0.009
Shunt dysfunction			
Previous splenectomy (yes/no)	1.76	1.16-2.67	0.008
Initial stent position (sub-optimal/optimal)	2.30	1.57-3.35	< 0.001
Survival			
Ascites (yes/no)	1.87	1.13-3.08	0.014
INR	2.31	1.34-3.98	0.003
Shunting branch of the PV (right/left)	1.92	1.30-2.84	0.001
Initial stent position (sub-optimal/optimal)	1.68	1.12-2.50	0.011
Child-Pugh score	1.27	1.13-1.43	< 0.001
MELD	1.09	1.03-1.14	0.003
HE within six months (yes/no)	1.54	1.02-2.35	0.043
Shunt dysfunction within 6 mo (yes/no)	2.48	1.47-4.19	0.001

INR: International normalized ratio; PV: Portal vein; MELD: Model for end-stage liver diseases; HE: Hepatic encephalopathy; PSG: Portosystemic pressure gradient.

0.029). In the 10-mm stent subgroup, the 3-year survival rates were 78.2% and 60.6% for the patients with O-SP and those with sub-O-SP, respectively (Figure 4F, log-rank test: $P = 0.164$).

Thirteen variables were identified as potential prognostic factors of survival in the univariate analyses (Table 2). A multivariate analysis showed that ascites ($P < 0.015$), INR ($P = 0.002$), Child-Pugh score ($P < 0.001$), MELD score ($P = 0.002$), the shunting branch of the PV ($P = 0.001$), the initial stent position ($P = 0.010$), HE within 6 mo ($P = 0.043$) and shunt dysfunction within 6 mo ($P = 0.001$) were independent predictors (Table 3).

DISCUSSION

This study of TIPS creation for variceal bleeding in a large cohort of cirrhotic patients verified that shunting the left PV may decrease the risk of HE and that the deployment of a stent with O-SP could reduce the incidence of shunt dysfunction. Most importantly, we revealed that the shunting branch of the PV and the initial stent position were independent prognostic factors of patient survival.

Recently, a decreased risk of HE after left PV TIPS creation was observed in a study of 72 patients[16] and consequently confirmed by a study with 80 patients[17]. However, the former study found no significant difference in the overall survival of patients with a TIPS placed in the left *vs* right PV[16], and the latter study did not address the survival of these two groups of patients[17]. The authors of the former study considered that the negative results were attributed to the short observation periods and the relatively small sample size of the study, as that they did not employ survival as the primary endpoint[16]. We confirmed that patients with a TIPS placed through the left PV have a lower risk of HE in a large cohort of patients. In our multivariate analysis, patients with HE within six months and the use of the right PV were found to be two of the independent risk factors for mortality. These results suggest that TIPS to the left PV may improve patient survival by lowering the risk of HE to a great degree. Survival is usually considered to be the strongest endpoint for evaluating the effectiveness of a therapy. Thus, our study presents important evidence for using the left PV in cirrhotic patients who underwent TIPS creation for variceal bleeding.

It is reported that most HE neurotoxins, such as ammonia, are derived from the intestine[27], which may be directed predominantly to the right PV[28,29]. Thus, TIPS placement through the right PV may theoretically lead

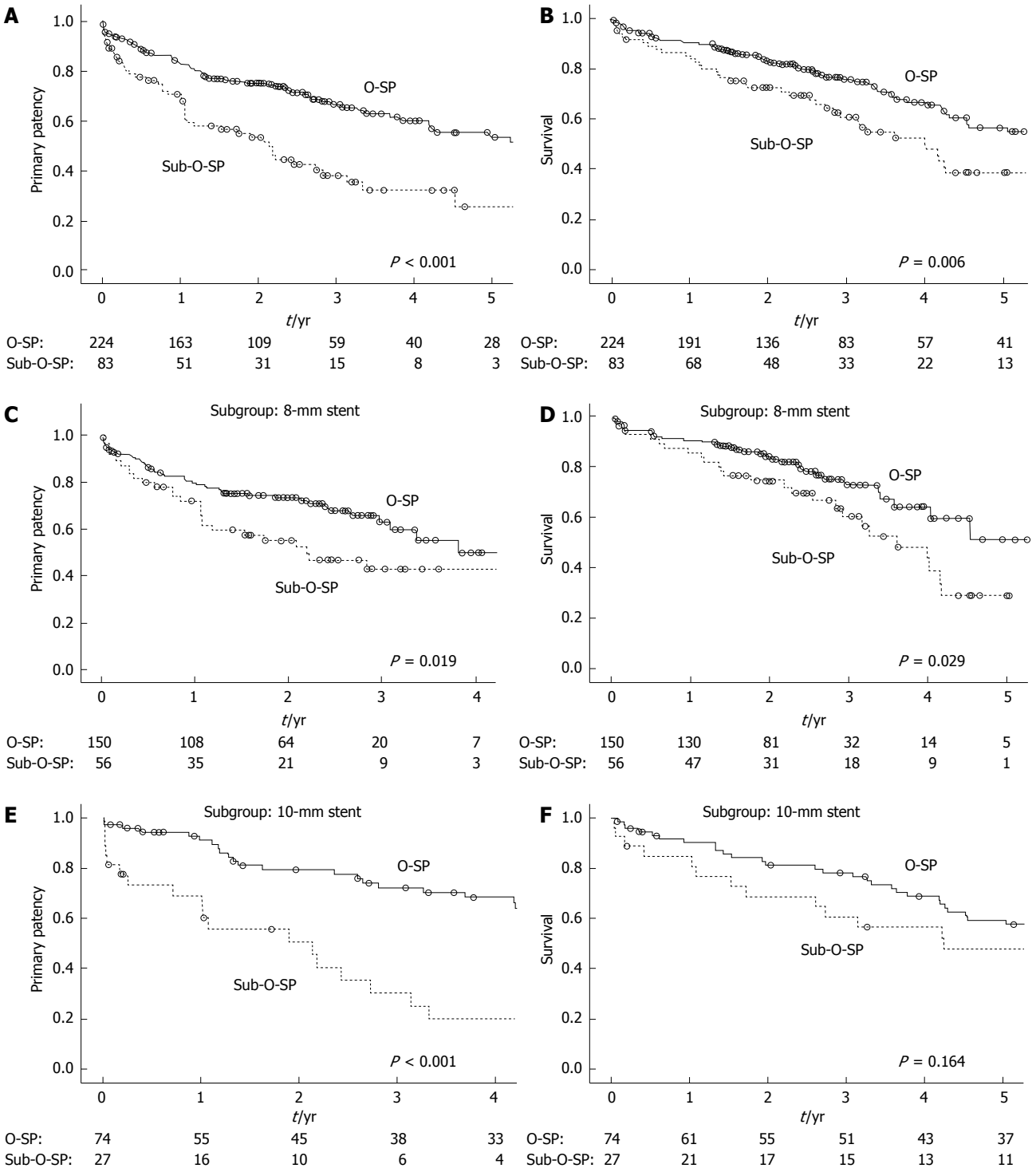
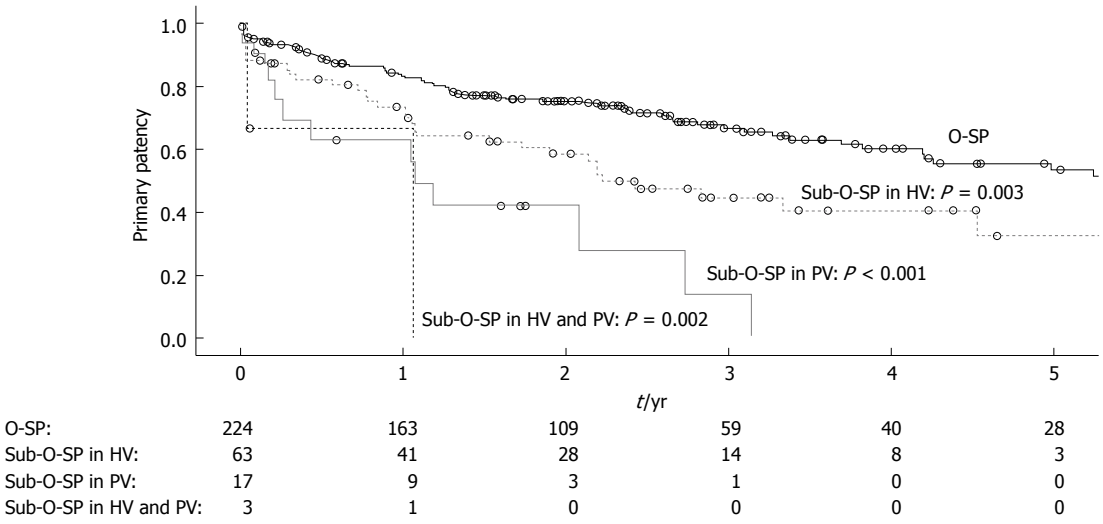


Figure 4 Patency results from the Kaplan-Meier analyses. Comparison of primary patency between patients with optimal initial stent position (O-SP) and those with sub-O-SP in all patients (A), an 8-mm stent subgroup (C) and a 10-mm stent subgroup (E). Comparisons of survival between the patients with O-SP and those with sub-O-SP in all patients (B), an 8-mm stent subgroup (D) and a 10-mm stent subgroup (F).

to increased neurotoxins in the systemic circulation, in which the effect will result in an increased incidence of HE and decreased survival[16,17].

One previous study demonstrated that patients with the cephalic end of the stent extended to the hepatocaval junction had a longer patency lifespan compared to those with a stent terminating in the HV; however, the authors did not assess the effect of the spatial relationship between the caudal end of the stent and the vascular wall of the PV on shunt patency[18]. Stenosis in the PV was also reported as an important cause of shunt dysfunction, especially in patients with covered stents[13,19,30-32]. In our 13 years' experience, we have the impression that failure of the caudal end of the stent to be parallel to the vascular wall of the PV probably increases the risk of stenosis in the PV. In order to validate our impression,



O-SP:	224	163	109	59	40	28
Sub-O-SP in HV:	63	41	28	14	8	3
Sub-O-SP in PV:	17	9	3	1	0	0
Sub-O-SP in HV and PV:	3	1	0	0	0	0

Figure 5 Patency results in patients with different stent positions. Comparison of primary patency between patients with optimal initial stent position (O-SP) and those with sub-O-SP in the hepatic vein (HV) only, sub-O-SP in the portal vein (PV) only and sub-O-SP in both the HV and PV.

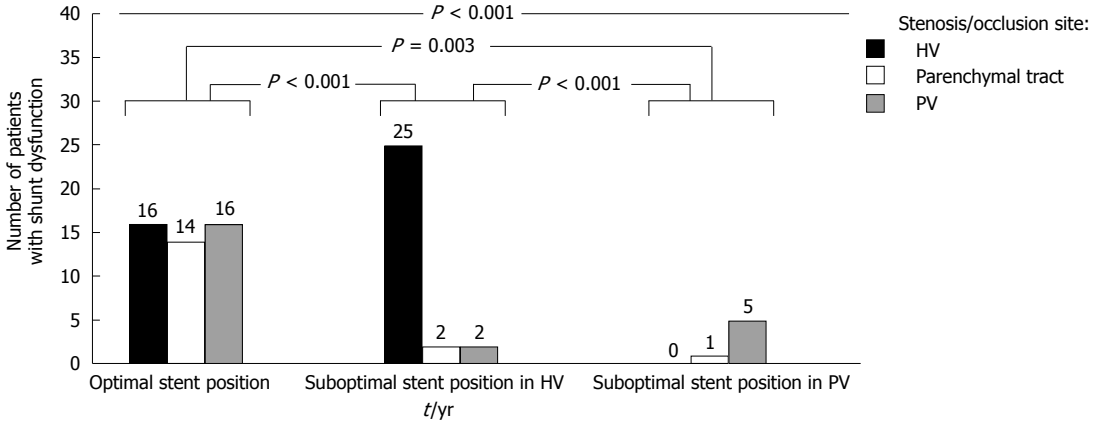


Figure 6 Relationship between stent positions and stenosis/occlusion sites. The stenosis/occlusion sites of the first shunt dysfunction among patients with optimal initial stent position (O-SP) in the hepatic vein(HV) and portal vein (PV), patients with sub-O-SP in the HV and patients with sub-O-SP in the PV are significantly different.

this suboptimal spatial relationship (the caudal end of the stent is unparallel to the vascular wall of the PV, Figure 1B) was defined as sub-O-SP in the PV and its effect on shunt dysfunction was studied in this study.

The high inter-observer agreement on the stent position classifications indicated that our classification criteria were reliable and valid. In this study, the patients with sub-O-SP in the HV and those with sub-O-SP in the PV had significantly lower primary patency rates compared to those with O-SP, and the outcomes of the three patients with sub-O-SP in both the HV and PV were notably worse. These results suggest that the deployment of a stent with the cephalic end extending to the hepato-caval junction and the caudal end parallel to the vascular wall of the PV is favorable in a TIPS procedure to decrease the risk of shunt dysfunction.

Additionally, the stenosis or occlusion sites in the patients with shunt dysfunction correlated well with their initial suboptimal stent sites. It has been reported that an uncovered HV is more susceptible to pseudointimal hyperplasia and more predisposed to shunt thrombosis caused by turbulence and the shear stress of high-velocity blood flow[33]. Moreover, in the patients with a stent terminating in the HV, uncovered outflow segments of the parenchymal tract are more likely to be formed by stent migration caused by organ movement[34]. For the patients with the caudal end of the stent failing to be parallel to the vascular wall of the PV, the chronic trauma to the PV intima caused by the end of the stent was reported to be responsible for the stenosis or occlusion in the PV[13,30].

Furthermore, our data demonstrate that patients with sub-O-SP had a significantly lower survival rate than those with O-SP. Moreover, the O-SP was revealed as an independent predictor of improved survival in the multivariate analysis. These results indicate that initial stent position plays an role in patient survival[13,18,19]. Subsequently, shunt dysfunction within six months was identified as one of the independent risk factors for survival, which suggests that the increased incidence of shunt dysfunction in patients with sub-O-SP may be partially responsible for the decreased survival rates. The higher shunt dysfunction rate determines many of the relevant

outcome parameters such as rebleeding, recurrence of ascites, hepatorenal syndrome, spontaneous bacterial peritonitis, hospitalization, and cost, most of which are also closely related to survival[35].

Compared to historical data in randomized studies, our patients demonstrated higher patency rates. This was also observed in our previous study on TIPS for PV thrombosis in cirrhosis[20]. It was reported in a randomized controlled trial that the routine administration of anticoagulants has a considerable effect on the improved patency rate[36]. Thus, the higher patency rate of our patients was partially attributed to the routine use of anticoagulants. To some degree, our study validated the effectiveness of anticoagulation on the prevention of shunt dysfunction. Another possible reason for our higher patency rate is that patients included in a randomized trial are more closely followed than in a retrospective study.

The retrospective design and long study duration are some of the limitations of this study. Modifications in the technical aspects of the TIPS procedure and improvements in the supportive care most likely occurred during a long-term study. However, in our study, the TIPS date was not identified as a risk factor for HE, shunt dysfunction, and mortality (Table 2), which means the patient outcome was relatively stable during the study period. Another limitation is the use of two different sized stents, which adds another confounding variable. However, the results of the subgroup analyses according to the diameter of the stent are consistent with the results of the analyses in the total patient population. These results suggest that the shunting branch of PV and the initial stent position are important when stent in either diameter is used. The use of an uncovered stent is also a limitation of this study because of the worldwide popularity of covered stents. Certainly, TIPS with a covered stent faces challenge of shunting either the left or right PV, and with the risk of having a sub-O-SP as well. To our knowledge, the effectiveness of the shunting branch of the PV and initial stent position on survival has yet not been studied in covered stents. Thus, the results of uncovered stents for TIPS creation will most likely have important reference value for the clinical practice and research of TIPS with covered stents.

In conclusion, this study of TIPS for variceal bleeding in cirrhotic patients found the following: (1) placement of a left PV TIPS decreases the risk of HE; (2) deployment of a stent with O-SP reduces the risk of shunt dysfunction; and (3) both of these factors may improve patient survival. The shunting of the left PV and the deployment of a stent with O-SP should be recommended for TIPS creation. Further prospective studies are needed to confirm these results.

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COMMENTS

Background

Transjugular intrahepatic portosystemic shunt (TIPS) is used worldwide for the prevention of variceal bleeding. Hepatic encephalopathy (HE) and shunt dysfunction are the major drawbacks of this procedure. Previous studies demonstrated that creation of a TIPS to the left portal vein (PV) instead of the right PV could decrease the risk of HE and that stent position is related to the occurrence of shunt dysfunction. However, the effects of the shunting branch of the portal vein and the initial stent position on patient survival were confused.

Research frontiers

More and more patients underwent the TIPS procedure for the complications of portal hypertension. For the use of TIPS procedure, the research hotspot is how to reduce the procedure-related complications and improve the patient survival by devising new devices, polishing the procedure technique, and bettering the patient selection.

Innovations and breakthroughs

Previous studies have found that patients with a TIPS placed to the left PV had lower risk of HE than those with a TIPS placed to the right PV. However, it is unclear whether the use of the left PV could improve patient survival. The results of our study validated the effect of creation of a TIPS to the left PV on the reduction of HE risk. Furthermore, we found that patients with a TIPS placed to the left PV had significantly better survival. Previously, several studies have assessed the effect of stent position on shunt patency rate. They demonstrated that patients with the cephalic end of the stent extended to the hepato-caval junction had a longer patency lifespan. All of these studies did not consider the positions of cephalic and caudal ends of the stent at the same time. In the present study, we defined the stent position as optimal initial stent position (O-SP) or non-O-SP by considering the positions of both the cephalic and caudal ends of the stent. The results demonstrated that patients with O-SP had better patency rate and long term survival.

Applications

The results of the present study suggest that further TIPS creation should be placed to the left PV with O-SP in both the cephalic and caudal ends.

Terminology

TIPS is an interventional procedure which created a shunt (the shunt is maintained with a metal stent) within the liver between the portal vein and hepatic vein to decompress the portal pressure. O-SP is a stent position that satisfied the following two criteria: (1) the cephalic end of the stent extended to the hepato-caval junction; and (2) the caudal end of the stent was parallel to the vascular wall of the PV (the angle between the tangent line of the caudal end of the stent and the vascular wall of the PV was less than 20°).

Peer review

This is an interesting study which retrospectively analyzed more than 300 patients who underwent TIPS procedure for variceal bleeding. The results are useful and suggest that further TIPS should be placed to the left PV with O-SP to improve the patient outcome.

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BRIEF ARTICLE

Predictors of survival in patients with established cirrhosis and hepatocellular carcinoma treated with sorafenib

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Abstract

AIM: To investigate in greater detail the efficacy and safety of sorafenib for the treatment of hepatocellular carcinoma (HCC) in patients with established cirrhosis.

METHODS: From October 2009 to July 2012 patients with an established diagnosis of cirrhosis and HCC treated with sorafenib were consecutively enrolled. According to the Barcelona Clinic Liver Cancer (BCLC) classification, patients were in the advanced stage (BCLC-C) or in the intermediate stage (BCLC-B) but unfit or unresponsive to other therapeutic strategies. Treatment was evaluated performing a 4-phase computed tomography or magnetic resonance imaging scan every 2-3 mo, and analyzed according to the modified Response Evaluation Criteria in Solid Tumors. Sorafenib was administered at 800 mg/d, until radiological progression or occurrence of unacceptable adverse events (AEs). Univariate and multivariate analyses identified predictors of 16-wk clinical benefit and overall survival.

RESULTS: Forty-four patients were enrolled, 15 had intermediate HCC and 14 a Child-Pugh score of B7. AEs caused treatment interruption in 19 patients (43%), and median treatment duration was shorter in this subset (5 wk *vs* 19 wk, $P < 0.001$) and in the BCLC-C subgroup (13 wk *vs* 40 wk, $P = 0.015$). No significant differences in the reason for treatment interruption or in treatment duration were found comparing patients in Child-Pugh class A *vs* B or in patients older or younger than 70 years. After 16 wk of treatment, 18 patients (41%) had stable disease or partial response. Patients with viral infection or BCLC-C were at higher risk of disease progression. ECOG, extrahepatic spread, macrovascular invasion, alpha-fetoprotein or alkaline phosphatase levels at admission were independent predictors of overall survival.

CONCLUSION: In patients with cirrhosis and HCC treated with sorafenib, AEs are a common cause of early treatment withdrawal. Vascular invasion and extrahepatic spread condition early response to treatment and survival. Baseline biochemical parameters may be helpful to identify patients at higher risk of shorter

overall survival.

Key words: Hepatocellular carcinoma; Sorafenib; Cirrhosis; Adverse events; Barcelona Clinic Liver Cancer

Core tip: The study provides information on the clinical characteristics and laboratory findings that predict survival in patients with hepatocellular carcinoma and established cirrhosis treated with sorafenib. This group of patients is particularly fragile and difficult to treat, and therapy with systemic agents, including sorafenib, requires careful monitoring. We report that parameters related to the tumor (extrahepatic spread, vascular invasion), common laboratory tests (alpha-fetoprotein or alkaline phosphatase) and patients characteristics (performance status) are significant predictors of overall survival in this group. These data provide important clinical information for the management of this type of patients.

Inghilesi AL, Gallori D, Antonuzzo L, Forte P, Tomcikova D, Arena U, Colagrande S, Pradella S, Fani B, Gianni E, Boni L, Laffi G, Di Costanzo F, Marra F. Predictors of survival in patients with established cirrhosis and hepatocellular carcinoma treated with sorafenib. *World J Gastroenterol* 2014; 20(3): 786-794 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i3/786.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i3.786

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most deadly cancers worldwide, and its incidence has increased steadily over the last 20 years in Western Europe and North America[1]. One of the hallmarks of HCC is its strict association with chronic liver disease and in particular cirrhosis, which represents the major risk factor for this type of cancer. The presence of cirrhosis makes the treatment of HCC particularly challenging, as tumor destruction must maximally preserve the amount of functioning liver tissue to prevent hepatic failure[1].

According to the Barcelona Clinic Liver Cancer (BCLC) classification, advanced HCC is identified as an unresectable HCC associated with symptoms, and/or macrovascular invasion, and/or extrahepatic spread[2]. While advanced HCC has long been considered untreatable, recent controlled trials have shown that sorafenib, a multi-kinase inhibitor, prolongs survival of these patients[3,4]. However, the survival benefit afforded by this drug is limited to a median of 8-10 wk over placebo, and information indicating which patients are more likely to take advantage from treatment with sorafenib is still very limited. Better patient selection would also be associated with rationalization of health care costs, given the high reimbursement price of sorafenib treatment[5]. Additionally, the burden of side effects associated with systemic therapies may be particularly heavy in patients with cirrhosis, who are prone to decompensation of the underlying disorder[6].

Although the safety of sorafenib has been the focus of some post-marketing studies, few have analyzed its effects in patients with a clear diagnosis of cirrhosis. Aim of this study was to evaluate the safety of sorafenib in patients with HCC and established cirrhosis, consecutively recruited in a single tertiary referral center, and to carefully analyze the variables that could be predictive of response to treatment or survival.

MATERIALS AND METHODS

Patients

From October 2009 to July 2012 all patients with hepatocellular carcinoma and an established diagnosis of cirrhosis undergoing treatment with sorafenib at one of the prescribing Centers at the Careggi Hospital, Florence, were enrolled. These included patients with advanced (stage C) HCC, according to BCLC classification[7] or with intermediate stage (BCLC-B) who were unfit or failed to respond to other approved therapeutic strategies. Patients were either referred to a group of gastroenterologists and hepatologists specifically dealing with the management of HCC (collectively defined as "Hepatology Unit") or to the Medical Oncology Unit of Careggi Hospital.

HCC was diagnosed by radiologic criteria according to American Association for the Study of Liver Diseases[8] or EASL guidelines[2] and/or by biopsy when required. In this study only patients with an established diagnosis of cirrhosis were included. Cirrhosis was diagnosed on the basis of a history of chronic liver disease, and clinical (presence of signs of portal hypertension, previous episodes of decompensation), imaging (liver nodular surface or splenomegaly), elastographic (stiffness $\geq$ 18 kPa) and/or hematological and biochemical findings (thrombocytopenia, hyperbilirubinemia, high International Normalized Ratio). Patients were sub-classified according to the Child-Pugh score[9]. All patients in whom a score of 7, but not higher, was calculated at least once in the two months preceding enrollment were considered as Child-Pugh class B, regardless of the actual score at the time of sorafenib initiation. Concomitant antiviral therapy for hepatitis B was allowed. Performance status was evaluated according to the Eastern Cooperative Oncology Group (ECOG)[10].

Treatment schedule and interruption, and dose modification

Sorafenib was administered at 800 mg/d. Adverse events (AEs) were graded according to the Common Terminology Criteria for Adverse Events (CTCAE) version 3.0[11]. Elevation of aminotransferases and/or bilirubin were collectively considered as "hepatic" AEs and graded according to CTCAE of the individual component. Appearance of AEs with a grade $\geq$ 2 resulted in dose re-

Table 1 Clinical characteristics of the patients with cirrhosis and hepatocellular carcinoma enrolled in the study *n* (%)

Characteristics	*n* = 44
Age (yr)	
mean (SD)	67.7 (10.1)
median (min-max)	70 (44-83)
Males	38 (86.4)
Recruiting unit	
Hepatology	29 (65.9)
Medical Oncology	15 (34.1)
Etiology of cirrhosis	
HCV	19 (43.2)
HBV	9 (20.5)
Alcohol	7 (15.9)
Multifactorial	4 (9.1)
Cryptogenic	4 (9.1)
Primary biliary cirrhosis	1 (2.3)
ECOG performance status	
0	24 (54.5)
1	18 (40.9)
2	2 (4.5)
BCLC	
B	15 (34.1)
C	29 (65.9)
Child-Pugh class	
A	29 (65.9)
B	15 (34.1)
Extrahepatic spread	9 (20.5)
Portal vein thrombosis	8 (18.2)
Varices	15 (34.1)
Macroscopic category	
Extrahepatic only	1 (2.3)
Uninodular	3 (6.8)
⩽ 3 nodules	34 (77.3)
> 3 nodules	6 (13.6)
Previous therapies	
TACE[1]	20 (45.5)
Locoregional ablation	3 (6.8)
Surgical resection	2 (4.5)
None	19 (43.2)

[1]Includes 7 patients in whom trans arterial chemio-embolization (TACE) was performed in combination with other treatment modalities, and 2 patients treated with ^{90}Y radio-embolization. HCV: Hepatitis C virus; HBV: Hepatitis B virus; ECOG: Eastern Cooperative Oncology Group; BCLC: Barcelona clinic liver cancer.

duction, temporary withdrawal or permanent interruption according to the physician's decision. Dose reduction or temporary interruption were maintained until AE resolution or grade regression, and was based on the physician's judgment. Therapy was continued until progression, death, or appearance of unacceptable AEs. Treatment was also stopped upon withdrawal of patient's consent.

Patient follow-up

Patients underwent evaluation at baseline and at weekly to monthly intervals, according to the physician's judgment. Blood tests were obtained at intervals not greater than 4 wk and included a complete blood count and serum chemistries. Treatment was evaluated performing a 4-phase computed tomography (CT) or magnetic resonance (MR) scan, and analyzed according to the modified Response Evaluation Criteria in Solid Tumors (mRECIST)[12]. Radiologic evaluation was repeated every 2-3 mo until progression or treatment interruption for AEs. Patients were followed after treatment suspension and overall survival was evaluated.

Statistical analysis

In descriptive statistics χ^2 test and Fisher's exact test were used for testing differences for categorical variables and Mann-Whitney *U* test for continuous variables between patient groups. A *P* value < 0.05 was considered statistically significant. Further analyses were targeted on: (1) 16-wk clinical benefit (*i.e.*, presence/absence of partial response or stable disease at imaging) using logistic regression; and (2) overall survival (end-point was death during follow-up, other patients were censored up to the date of the last contact) using Kaplan-Meier method, log-rank test and Cox PH modeling.

In both types of analyses all clinical variables collected at enrolling were considered. The frequency of missing values ranged from 0% to 13.6%, mostly present in laboratory parameters. Due to the small number of patients in the sample, missing values were handled by single imputation technique. The results coming from univariate analyses before and after data imputation were compared. Parameters resulting statistically significant with *P* < 0.20 in univariate analyses were further included into multivariate analyses. Then, starting from the full model with all variables included, the models were built applying backwards stepwise selection procedure with *P* > 0.10 as remove and *P* < 0.10 re-entry criteria. As a strength of association between each predictor and outcome the OR and 95%CI for logistic regression and HR, and 95%CI for survival analysis were calculated. Statistical analysis was performed with SAS 9.2 software (SAS Institute Inc., Cary, NC, United States).

RESULTS

The clinical characteristics of the 44 patients enrolled in the study are shown in Table 1. Patients were mostly males and their age concentrated in the 7th-8th decade. Viral etiology, alone or in combination with other factors, was present in more than two-thirds of patients. Nine patients had extrahepatic spread (4 lungs, 3 lymph nodes and 2 skeleton). The majority of patients (84%) had a tumor burden ⩽ 3 nodules. Approximately half of the patients (56.8%) had been previously treated with other modalities, chemoembolization being the most frequent. No patient had undergone liver transplantation. No patient was lost to follow up during treatment, while 2 patients were lost to follow up after sorafenib withdrawal.

Median treatment duration was 15 wk (range 1-81). At the time of analysis no patients were still under treatment. The reason for stopping treatment was disease progression in 25 patients (56.8%) and unacceptable AEs in 19 (43.2%). Median treatment duration was significantly shorter in patients who stopped treatment because of AEs [5 (range 1-57) *vs* 19 (5-81) wk, *P* < 0.001]. No significant differences in the reason for treatment interruption or in treatment duration were found comparing patients

Table 2 Incidence of adverse events in 44 patients with cirrhosis and hepatocellular carcinoma undergoing treatment with sorafenib *n* (%)

Adverse events	Statistics
Fatigue	29 (65.9)
Grade 1	11 (25.0)
Grade 2	11 (25.0)
Grade 3	7 (15.9)
Bleeding	7 (15.9)
Grade 1	3 (6.8)
Grade 2	1 (2.3)
Grade 3	3 (6.8)
Hand-foot syndrome	14 (31.8)
Grade 1	7 (15.9)
Grade 2	7 (15.9)
Grade 3	0 (0.0)
Diarrhea	15 (34.1)
Grade 1	6 (13.6)
Grade 2	8 (18.2)
Grade 3	1 (2.3)
Hepatic grade 3[1]AEs	8 (18.2)
Other AEs[2]	20 (45.5)

[1]An increase in aminotranspherase and bilirubin after starting sorafenib was observed in all patients; [2]Including rash, hypertension, alopecia, diabetes, mucositis, abdominal pain, voice changes. All grades were evaluated according to Common Terminology Criteria for Adverse Event version 3.0. AEs: Adverse events.

in Child Pugh A *vs* B class or patients older or younger than 70 years. Patients in the BCLC-C stage had a significantly shorter median duration of treatment compared to BCLC-B patients [13 (2-73) *vs* 40 (1-81) wk, $P = 0.015$). However, these two latter groups did not differ considering the causes of treatment interruption. Interestingly, patients enrolled in the Oncology Unit were more likely to interrupt treatment due to AEs than patients enrolled in the Hepatology Unit (73.3% *vs* 27.6%, $P = 0.01$).

The overall incidence of AEs during the treatment period was 93.2%. As shown in Table 2, fatigue was the most frequently observed AE, and occurred in 2 out of 3 patients. Twenty-eight patients (63.7%) presented 2 or more AEs during treatment, irrespective of the grade. Remarkably, 25% and 11% of the whole series presented 3 or 4 AEs, respectively, and in one case (2.3%) 5 AEs were recorded. Grade 3 AEs were observed in 19/44 patients (43.2%), but no more than one grade 3 AE was observed in the same patient. In the 19 patients who stopped treatment because of AEs, grade 3 fatigue (7 patients) or hepatic AEs (8 patients) were those most frequently involved. Twenty-one patients (47.7%) required dose reduction or temporary interruption of sorafenib treatment. Considering the whole series, 28 patients (63.6%) received a mean daily dose of sorafenib greater than 400 mg. After excluding those patients whose treatment duration was lower than 4 wk, 24 out of 37 patients (64.9%) received more than 400 mg/d sorafenib. No significant differences in the overall occurrence of AEs was found comparing patients in Child A *vs* B classes. Grade 3 hepatic AEs tended to be more common in Child B patients, although this difference did not reach statistical significance (33.3% *vs* 10.3%, $P = 0.099$). AEs occurred at a similar frequency comparing patients older or younger than 70 years or those in BCLC-C *vs* B stage. Biochemical parameters at the start and at the end of sorafenib therapy are shown in Table 3, subdivided according to the reason for interruption.

To identify possible predictors of the clinical response to sorafenib, we analyzed our series of patients after 16 wk of sorafenib treatment, defining a group with "clinical benefit", as the composite of stable disease or partial response by CT/MR dynamic imaging. This group was compared to patients who showed progressive disease at this or earlier time points, or who interrupted treatment because of AEs. At the time of this evaluation, no complete responses were recorded, and no patients had died. Eighteen out of 44 patients (40.9%) had a clinical benefit at week 16, with 14 (31.8%) showing stable disease and 4 (9.1%) a partial response. Eleven patients (25%) had stopped because of a radiological progression and 15 (34.1%) due to the appearance of unacceptable AEs. Univariate analysis of the parameters associated with clinical benefit demonstrated that ECOG performance status 0, cirrhosis of non-viral etiology and BCLC-B stage of disease were significantly associated with a clinical benefit at week 16 (Table 4). In a multivariate model, only BCLC-B and the presence of non-viral cirrhosis emerged as independent predictors of clinical benefit after 16 wk of treatment (Table 4).When a sub-analysis comparing patients infected with Hepatitis C virus (HCV) or Hepatitis B virus (HBV) was performed, no significant differences in survival were observed.

Median overall survival was 11.4 mo (range 7.1-15.7) (Figure 1A). At the time of analysis, 8 patients were still alive, none of them continuing sorafenib. In univariate analysis we found that ECOG performance status $\geq$ 1, presence of metastases or macrovascular invasion, BCLC-C stage, and elevated baseline levels of alpha-fetoprotein (AFP) or alkaline phosphatase (ALP) were significantly related to a shorter overall survival (Table 5). The multivariate model (Table 6) indicated that the presence of symptoms (ECOG performance status $\geq$ 1), macrovascular invasion, high levels of ALP or AFP at admission were independent predictors of mortality. Figure 1B and C show Kaplan-Meier curves for the series of patients stratified according to AFP or ALP levels, respectively.

DISCUSSION

Sorafenib is the only systemic therapy approved for HCC in its advanced stage and has also been proposed for patients with otherwise untreatable, intermediate stage HCC[13]. In this study, we report data from a group of patients with HCC and a definite diagnosis of cirrhosis, consecutively recruited in single, tertiary referral center. Tolerability of sorafenib treatment was slightly lower than the one reported in the registration studies, in particular the SHARP trial (93% *vs* 80% incidence of AEs)[3], and

Table 3 Changes in laboratory parameters at the beginning and the end of sorafenib treatment according to the reason of treatment interruption

			Bilirubin (mg/dL)	gGT (U/L)	ALP (U/L)	Platelets/mm^3	INR	AST (U/L)	AFP (ng/mL)
Patients with treatment suspension due to progressive disease (n = 25; median treatment duration 19 wk)	Baseline	mean (SD)	1.3 (0.8)	192.5 (187.5)	173.8 (92.5)	143160 (83075.6)	1.18 (0.25)	66.5 (35.7)	3668.6 (8533.3)
		med (min-max)	1.2 (0.2-3.5)	114 (41-648)	142 (64-436)	125000 (50000-337000)	1.10 (0.9-2.2)	66 (3.6-191)	18.8 (2.7-28136)
	End of treatment	mean (SD)	2.3 (1.7)	256.4 (276.8)	225.4 (196.3)	151360 (93196.4)	1.17 (0.26)	106.4 (97.8)	11060.4 (21903)
		median (min-max)	1.6 (0.3-6.4)	165 (20-1249)	182 (75-1048)	114000 (42000-426000)	1.1 (0.9-2.2)	71 (2-472)	83.4 (3.0-73434.5)
	Mann-Whitney test	P value	0.003	0.008	0.163	0.391	0.724	0.012	0.013
Patients with treatment suspension due to AEs (n = 19; median treatment duration 5 wk)	Baseline	mean (SD)	1.6 (0.7)	144.4 (107.4)	170 (83)	122125 (67280.4)	1.14 (0.92)	76.0 (38.6)	1739.0 (2841.1)
		med (min-max)	1.4 (0.6-3.1)	123 (40-416)	159.5 (65-345)	102000 (35000-254000)	1.1 (1.0-1.3)	66.5 (27-188)	70.6 (1.6-6836.9)
	End of treatment	mean (SD)	2.9 (2.8)	168.3 (147.1)	218.8 (142.8)	135500 (75371.1)	1.14 (0.17)	91.1 (41.5)	2204.4 (4406.1)
		median (min-max)	2.3 (0.7-12.2)	121.5 (40-525)	183 (75-515)	123000 (37000-277000)	1.05 (1.0-1.4)	83.5 (27-153)	124.1 (2.1-14000)
	Mann-Whitney test	P value	0.029	0.441	0.051	0.147	0.891	0.125	0.333

gGT: Gamma-glutamyltranspeptidase; ALP: Alkaline phosphatase; AST: Aspartate transaminase; AFP: Alpha-fetoprotein; INR: International normalized ratio.

Table 4 Univariate and multivariate analysis of the factors associated with a 16-week "clinical benefit" as defined by the presence of partial response or stable disease at imaging, according to modified Response Evaluation Criteria in Solid Tumors criteria

Variable	Univariate analysis		Multivariate analysis	
	OR (95%CI)	P value	OR (95%CI)	P value
ECOG				
0	1 (ref.)	0.013	[2]	[2]
1-2	0.18 (0.05-0.70)			
Cirrhosis etiology				
Non-viral	1 (ref.)	0.040	1 (ref.)	0.043
HCV or HBV[1]	0.23 (0.06-0.94)		0.21 (0.05-0.95)	
HCV infection				
Absent	1 (ref.)	0.032	[2]	[2]
Present	0.24 (0.07-0.88)			
BCLC class				
B	1 (ref.)	0.003	1 (ref.)	0.004
C	0.12 (0.03-0.48)		0.10 (0.02-0.49)	
Previous therapy				
No	1 (ref.)	0.172	[2]	[2]
Yes	0.42 (0.12-1.45)			
AFP (ng/mL)				
≤ 400	1 (ref.)	0.069	[2]	[2]
> 400	0.28 (0.07-1.10)			

[1]Including patients with other concurrent etiologies; [2]Removed during the stepwise variable selection, not included in the final multivariate model. AFP: Alpha-fetoprotein; HCV: Hepatitis C virus; HBV: Hepatitis B virus; ECOG: Eastern Cooperative Oncology Group; BCLC: Barcelona clinic liver cancer.

was similar to other field practice studies[13]. It should be kept in mind that sorafenib is a non-curative treatment, and therefore maintenance of the best possible quality of life should always be considered as an essential target in these patients[5,14]. We found that the impact of AEs was more marked than in the SHARP trial, because fatigue was complained by two thirds of our patients, and in one fourth of these it reached grade 3 severity. In all patients who complained of fatigue, this symptom had a major influence on the quality of life, and was the cause of dose reduction in several cases. We also observed a higher incidence of grade 3 hepatic AEs, which are particularly worrisome in patients with cirrhosis such as the ones investigated in this study. It is important to emphasize that almost half of the whole group of patients (19/44) could not continue therapy due to intolerable AEs, and in 15 out of these 19 patients (78.9%) treatment interruption was related to grade 3 fatigue or hepatic AEs. Of note, we observed a significantly shorter median duration of treatment in patients who interrupted treatment due to AEs. This may reflect on patient management, as close follow-up with office visits together with early and aggressive symptomatic therapy may maximize the possibility to drive the patient through this initial critical phase of the therapy. On the other hand, a prompt recognition of initial signs of hepatic AEs may avoid an eventual and possibly fatal deterioration of liver function.

A somehow surprising finding of this study is the lack of difference in terms of AEs incidence or AE-related treatment interruption comparing Child A *vs* Child B patients. These results may be at least partially dependent on the low number of patients and on the fact that all Child-Pugh B patients were extremely well compensated. Nonetheless, Child-Pugh B patients may still have a higher propensity to deteriorate hepatic function, as a trend towards a higher frequency of severe hepatic AEs was observed. Together with data available in the literature[15-20], the results of the present study indicate that sorafenib therapy could be reasonably proposed to 'borderline' Child Pugh B patients, such as the ones described herein. However, close monitoring by a multidisciplinary group involving a Hepatologist is critical, as suggested by the observation that cirrhotic patients enrolled in an Oncology Unit were more likely to interrupt treatment due to AEs, although this did not translate into any differ-

Table 5 Univariate analysis of factors associated with overall survival in 44 patients with hepatocellular carcinoma and cirrhosis treated with sorafenib

Variable	*n*	Overall survival	
		HR (95%CI)	*P* value
Gender			
Male	38	1 (ref.)	0.437
Female	6	1.52(0.53-4.38)	
ECOG			
0	24	1 (ref.)	0.049
1-2	20	2.01(1.01-4.05)	
Age, yr			
< 70	22	1 (ref.)	0.297
⩾ 70	22	1.43 (0.73-2.78)	
Cirrhosis etiology			
Non-viral	12	1 (ref.)	0.832
HCV or HBV[1]	32	1.09 (0.51-2.34)	
Extrahepatic spread			
Absent	35	1 (ref.)	0.049
Present	9	2.26 (1.01-5.10)	
Portal thrombosis			
Absent	36	1 (ref.)	0.043
Present	8	2.52 (1.03-6.16)	
HBV infection			
Absent	32	1 (ref.)	0.279
Present	12	0.64 (0.29-1.43)	
HCV infection			
Absent	23	1 (ref.)	0.423
Present	21	1.32 (0.67-2.57)	
Esophageal varices			
Absent	28	1 (ref.)	0.883
Present	16	1.06 (0.52-2.14)	
Child Pugh score			
A	29	1 (ref.)	0.085
B	15	1.98 (0.91-4.29)	
BCLC class			
B	15	1 (ref.)	0.007
C	29	2.89 (1.34-6.25)	
Previous therapy			
No	19	1 (ref.)	0.526
Yes	25	1.25 (0.63-2.49)	
Bilirubin (mg/dL)			
⩽ 1.5	27	1 (ref.)	0.124
>1.5	17	1.76 (0.86-3.60)	
gGT (U/L)			
⩽ 48	5	1 (ref.)	0.483
> 48	39	1.54 (0.46-5.14)	
ALP (U/L)			
⩽ 120	13	1 (ref.)	0.013
>120	31	3.20 (1.28-7.98)	
Platelets/mm^3			
⩽ 150000	28	1 (ref.)	0.317
> 150000	16	0.69 (0.33-1.43)	
AST (U/L)			
⩽ 40	9	1 (ref.)	0.388
> 40	35	1.52 (0.59-3.94)	
AFP (ng/mL)			
⩽ 40	22	1 (ref.)	0.016
> 40	22	2.38 (1.18-4.80)	
AFP (ng/mL)			
⩽ 400	27	1 (ref.)	0.017
> 400	17	2.38 (1.17-4.86)	
Interruption due to disease progression			
No	19	1 (ref.)	0.238
Yes	25	0.67 (0.34 - 1.31)	

[1]Including patients with other concurrent etiologies. gGT: Gamma-glutamyltranspeptidase; ALP: Alkaline phosphatase; AST: Aspartate transaminase; AFP: Alpha-fetoprotein; HCV: Hepatitis C virus; HBV: Hepatitis B virus; ECOG: Eastern Cooperative Oncology Group; BCLC: Barcelona clinic liver cancer.

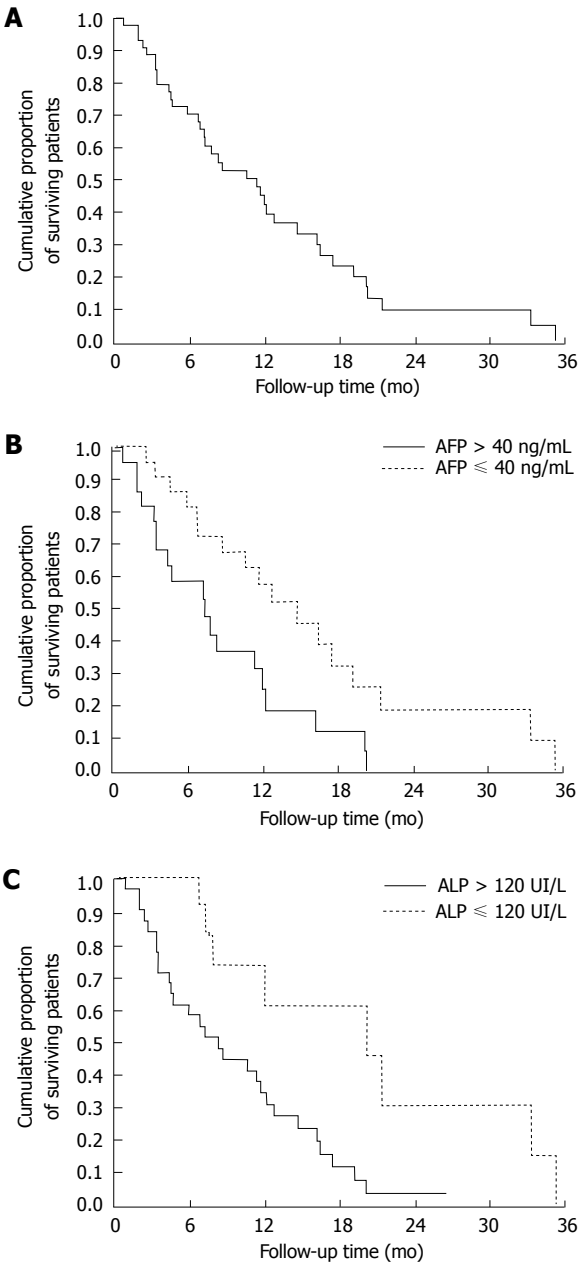

Figure 1 Kaplan-Meier survival curves of patients enrolled in this study. Forty-four patients with hepatocellular carcinoma and established cirrhosis were enrolled and treated with sorafenib. A: Overall survival; B: Survival of patients stratified according to their baseline alpha-fetoprotein (AFP) levels (P = 0.021); C: Survival of patients stratified according to their baseline alkaline phosphatase (ALP) levels (P = 0.017).

ences in survival. Interestingly, we did not find any differences in the overall appearance of AEs in a sub-analysis performed according to the age of the patients (below or above 70 years), in agreement with data from a Korean study[21]. This is particularly relevant, as in Western countries HCC is increasingly observed in the elderly[22].

A common clinical problem is the need to inform patients on the possible outcomes of sorafenib therapy in the medium term. For this reason, we analyzed the "clinical benefit" afforded by sorafenib treatment at 16 wk as a composite of radiological stable disease or partial response, versus treatment interruption regardless of its

Table 6 Multivariate analysis of overall survival in 44 patients with hepatocellular carcinoma and cirrhosis treated with sorafenib

Variable	OS		
	HR (95%CI)	Median OS in weeks (range)	*P* value
ECOG			
0	1 (ref.)	50.2 (9.3-153.1)	0.031
1-2	2.36 (1.08-5.16)	29.0 (2.9-92.6)	
Extrahepatic spread			
Absent	1 (ref.)	45.9 (8.1-153.1)	0.059
Present	2.41 (0.97-6.01)	19.1 (2.9-83)	
Portal thrombosis			
Absent	1 (ref.)	45.8 (2.9-153.1)	0.015
Present	3.33 (1.27-8.72)	29.9 (8.1-50)	
ALP (U/L)			
≤ 120	1 (ref.)	45.9 (16.6-153.1)	0.017
> 120	3.13 (1.23-8.00)	33.2 (2.9-115.4)	
AFP (ng/mL)			
≤ 40	1 (ref.)	52.5 (10.7-153.1)	0.021
> 40	2.33 (1.13-4.78)	31.0 (2.9-87.4)	

OS: Overall survival; ECOG: Eastern Cooperative Oncology Grou; ALP: Alkaline phosphatase; AFP: Alpha-fetoprotein.

reason. This provides an indication of sorafenib efficacy at a time when the impact of early withdrawal due to AEs could be outbalanced by benefit in the subsequent weeks. Intermediate stage, and non-viral etiology of cirrhosis were independent predictors of clinical benefit at week 16 of treatment. These data support the potential usefulness of sorafenib in selected BCLC-B patients, in line with recent evidence reported in a sub-analysis of the SHARP trial or in field-practice studies[13,23]. The possible significance of the predictive role of a non-viral etiology remains speculative, although in another, larger study the time to progression in HBV infected patients was not affected by sorafenib treatment[23]. Both HBV and HCV have been shown to interact with the Raf pathway and/or to modulate angiogenesis[24-27]. As these biologic actions are two of the major targets of sorafenib, viral infection may translate in a lower sensitivity to treatment. Although cirrhosis caused by HBV or HCV has generally different outcomes and prognosis, we found no significant differences in clinical benefit and overall survival comparing these two groups of patients. This result could be related to the small number of patients enrolled, although at this time no clear associations between the type of viral infection and the outcome of HCC patients treated with sorafenib have been reported.

Median overall survival of the patients included in the present study was comparable to the one of the SHARP study and of the largest field-practice studies published so far[3,13,15], indicating that selection of the patients was similar to what reported by other groups treating patients with advanced HCC. Macrovascular invasion and extrahepatic spread were strong predictors of survival, as reported in other studies[13,17,28], confirming the prognostic significance of the BCLC classification, where these parameters characterize transition from stage B to C. Moreover, a performance status greater than 0 was associated with a 2-fold greater risk of shorter overall survival, in agreement with data reported by the SOFIA group[13]. However, when evaluating the significance of performance status in HCC, the presence of cirrhosis should be considered, which by itself has an impact on the quality of life of the patient[29].

Increased AFP baseline levels were found to confer a risk of mortality 2.33 times higher than patients below that limit. A possible role of AFP as a predictor of mortality in HCC has been reported in other field practice studies[3,15,18,21], but the observed cut-off values were usually in a higher range. AFP levels have been shown to be associated with activation of the progenitor cell compartment and to correlate with a 'hepatoblastoma' signature in transcriptomic studies[30]. Similar considerations may be made for the role of ALP, because patients with abnormal levels had a more than three times higher risk of shorter survival at multivariate analysis. ALP levels have been reported to predict survival in patients treated with different modalities[31-33], although its role during systemic therapy has not been completely established. The pathophysiologic significance of this parameter remains uncertain, although it may be related to invasion of the smaller bile ducts as expression of the tendency of HCC to infiltrate adjoining structures. Taken together, data related to AFP and ALP indicate that simple baseline biochemical parameters may help to frame the patient in the most appropriate prognostic group.

Several limitations of the present study should be acknowledged, particularly the relatively small number of patients enrolled. On the other hand, although other studies have been published since sorafenib approval in 2008, in few cases a detailed analysis of patients with established cirrhosis associated with HCC has been conducted. Another limitation is related to the lack of histologic biomarkers to be possibly correlated with clinical outcomes. The fact that in cirrhotic patients imaging studies are usually sufficient to make a diagnosis of HCC[2] has considerably limited the use of biopsy, an invasive procedure, but has hindered the discovery of molecular factors associated with prognosis. Although no molecular predictors of the response to sorafenib have yet been identified, additional investigation is warranted to try to select those patients who are more likely to benefit from a therapy which is expensive and sometimes difficult to tolerate.

In conclusion, in patients with cirrhosis and HCC treated with sorafenib, early management of AEs is critical, as they present in the large majority of patients and are a common cause of early withdrawal of the treatment. Tumor vascular invasion and extrahepatic spread are the most relevant factors conditioning early response to treatment and survival. Moreover, tumor burden rather than parameters of liver function is critically relevant in the prognosis of these patients. Finally, common baseline biochemical parameters, such as AFP and ALP allow to identify patients at higher risk of a shorter overall survival.

COMMENTS

Background

Sorafenib has been recently approved for the treatment of hepatocellular carcinoma, but only limited information is available on its effects in conditions of everyday practice. This is particularly true when patients with a clear diagnosis of cirrhosis are considered. Due to the high costs of sorafenib therapy and the burden of side effects, it is very important to obtain information on the predictors of survival and of clinical response in patients treated with this drug, especially in the presence of established cirrhosis.

Research frontiers

The registration studies showing the efficacy of sorafenib in prolonging survival of patients with advanced hepatocellular carcinoma have given a great impulse to further research trying to better define which patients have a higher likelihood to benefit from treatment with this drug. This topic has a high clinical relevance and generates major efforts.

Innovations and breakthroughs

Identification of the predictive parameters of the response to sorafenib has been the focus of several studies after approval of this drug. Cirrhotic patients are extremely fragile and difficult to treat, and therapy with systemic agents should be conducted with great care. The authors found that parameters related to the tumor (extrahepatic spread, vascular invasion), and the patient (*e.g.*, laboratory tests including alpha-fetoprotein or alkaline phosphatase, or the patient's performance status) are predictors of overall survival in this group.

Applications

Data presented in this study provide useful clinical information for the management of cirrhotic patients with advanced hepatocellular carcinoma.

Terminology

Hepatocellular carcinoma is the most prevalent primary liver tumor and is notoriously difficult to treat. It is associated in the great majority of cases with chronic liver injury or cirrhosis. Sorafenib is a multi-kinase inhibitor approved for the treatment of advanced hepatocellular carcinoma. It acts both limiting tumor-associated angiogenesis and tumor cell proliferation.

Peer review

In this manuscript, the Authors reported the efficacy and safety of sorafenib for hepatocellular carcinoma in patients with cirrhosis, and analyzed biomarker as predictors of outcomes. The results obtained seem to be reasonable and have some new information. The paper is well written, although there are some drawbacks, particularly the fact that the size of study is small.

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BRIEF ARTICLE

Bortezomib effect on E2F and cyclin family members in human hepatocellular carcinoma cell lines

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Abstract

AIM: To evaluate the effects of the proteasome inhibitor bortezomib (BZB) on E2Fs and related genes in hepatocellular carcinoma (HCC) cells.

METHODS: The mRNA levels of the E2F family members (pro-proliferative: E2F1-3 and anti-proliferative: E2F4-8) and of their related genes cyclins and cyclin-dependent kinases (*cdks*) were evaluated in two HCC cell lines following a single BZB administration. mRNA levels of the epithelial-mesenchymal transition (EMT) genes were also measured in both cell lines after BZB treatment. The BZB concentration (40 nmol/L) used was chosen to stay well below the maximal amount/cm^2 recommended for *in vivo* application, and 2 d incubation was chosen as this time point has been found optimal to detect BZB effects in our previous studies. The HCC cell lines, HepG2 and JHH6, were chosen as they display different phenotypes, hepatocyte-like for HepG2 and undifferentiated for JHH6, thus representing an *in vitro* model of low and high aggressive forms of HCC, respectively. The mRNA levels of the target genes were measured by two-color microarray-based gene expression analysis, performed according to Agilent Technologies protocol and using an Agilent Scan B. For the E2F family members, mRNA levels were quantified by real-time reverse transcription polymerase chain reaction (RT-PCR). Using small interfering RNA's, the effects of E2F8 depletion on cell number was also evaluated.

RESULTS: After BZB treatment, microarray analysis of the undifferentiated JHH6 revealed a significant decrease in the expression of the pro-proliferative E2F member E2F2. Quantitative RT-PCR data were in keeping with the microarray analysis, and showed a significant increase and decrease in E2F8 and E2F2 mRNA levels, respectively. In contrast, BZB treatment of the hepatocyte-like HCC cell line HepG2 had a significant impact on mRNA levels of 5 of the 8 E2F members. In particular, mRNA levels of the pro-proliferative E2F members E2F1, E2F2, and of the anti-proliferative member E2F8, decreased over 80%. Notably, a reduction in E2F8 expression in HepG2 and JHH6 cells following siRNA treatment had no impact on cell proliferation. As observed with JHH6, BZB treatment of HepG2 cells induced a significant increase in mRNA levels of an anti-proliferative E2F member, E2F6 in this case. As was observed with E2F's, more dramatic changes in mRNA levels of the E2F related genes cyclins and Cdks and EMT genes were observed after BZB treatment of HepG2 compared to JHH6.

CONCLUSION: The differential expression of E2Fs and related genes induced by BZB in diverse HCC cell phenotypes contribute to bortezomib's mechanism of action in hepatocellular carcinoma.

Key words: Bortezomib; Cyclins; E2F family; Hepatocellular carcinoma; Liver; Microarray; 26S proteasome

Core tip: The 26S proteasome inhibitor bortezomib has been proposed as a novel therapeutic molecule for hepatocellular carcinoma (HCC), being able to reduce cell growth. Little information is available on the effect of bortezomib (BZB) on many of E2Fs, a family of transcription factors regulating normal and tumor cell proliferation. Our data show, for the first time, the BZB effect on expression of E2F family members in HCC cell lines is not limited to the most studied E2F1, but, it extends also to other E2F members, in particular E2F2, E2F8 and E2F6, and the effect is phenotypic dependent.

Baiz D, Dapas B, Farra R, Scaggiante B, Pozzato G, Zanconati F, Fiotti N, Consoloni L, Chiaretti S, Grassi G. Bortezomib effect on E2F and cyclin family members in human hepatocellular carcinoma cell lines. *World J Gastroenterol* 2014; 20(3): 795-803 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i3/795.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i3.795

INTRODUCTION

Hepatocellular carcinoma (HCC), accounting for more than 90% of primary liver cancers, is a global health problem[1-4] as it represents the sixth most common cancer and the third cause of cancer related death worldwide. The HCC incidence is age-related with a peak at 70 years. However, variation in different populations has been observed. For example, in Japan the highest incidence is between 70-79 years, whereas in Chinese and black African populations the age of appearance is younger. Males are more often affected than women with an estimated ratio of 2.4. HCC occurrence is highest in East Asia, sub-Saharan Africa, and Melanesia (85% of cases); in developed countries the frequency is lower with the exception of Southern Europe which has a significantly higher frequency compared to other developed countries. The most common risk factors for HCC development are represented by chronic viral hepatitis (types B and C), alcohol intake and aflatoxins exposure.

HCC is usually diagnosed at an advanced stage when the affected patients are often no longer eligible for curative treatments, such as liver resection, liver transplant, or local radiofrequency ablation. The efficacy of systemic chemotherapy is also limited due to the resistance of this disease to anticancer agents[5]. At the moment, the only systemic therapy showing a significant prolonged patient survival is based on the use of sorafenib. This drug is able to inhibit a number of kinases including Raf-1, c-Kit, and the pro-angiogenic receptor tyrosine kinases vascular endothelial growth factor receptor, platelet derived growth factor receptor and fibroblast growth factor receptor 1, all involved in HCC progression and overall prognosis[6]. However, sorafenib only modestly improves patient survival prolonging life span approximately of three months[7]. Thus, the development of novel therapeutic approaches to treat HCC-affected patients is urgently required.

In the last decade the drug bortezomib (BZB) has been studied as a possible novel therapeutic treatment for HCC. BZB is a boronic acid dipeptide derivative able to inhibit the 26S proteasome[8]. In particular, the boron atom present in BZB is responsible for the specific and efficient binding to the catalytic site of the 26S proteasome. This molecular machine is responsible for the degradation, *via* the ubiquitin proteasome pathway, of proteins involved in cell differentiation, apoptosis and cell cycle regulation including cyclins, cyclin-dependent kinase inhibitors and tumor suppressor proteins. BZB induces the inhibition of the 26S proteasome leading to the increase in levels of various proteins which lead to the generation of confounding signals that promote cell cycle arrest and the activation of the apoptotic program. So far the use of BZB is indicated for the treatment of multiple myeloma and relapsed mantle cell lymphoma[8].

BZB use in HCC is under evaluation as shown by a recent phase Ⅱ clinical trial[9]. Interestingly, normal hepatocyte function is largely unaffected by BZB treatment, opening the possibility that this drug may not have important side effects in patients, at least when administered locally[10,11]. The mechanisms underlying BZB's actions are complex and not completely understood. BZB is able to down-regulate HCC cell migration and invasion[12] by suppressing focal adhesion kinase expression[13]. It also promotes apoptosis[10,14] by reducing p-Akt levels[15-17] and it can induce autophagy *via* proteasome independent mechanisms[18]. With regard to the cell cycle, BZB has been shown to down-regulate HCC cell proliferation by increasing the levels of the cell cycle inhibitors p27/p21[12,19,20], and reducing the levels of cyclin D1[11,20], the phosphorylated form of the retinoblastoma protein pRB, and the transcription factor E2F1[11,20].

E2F1 belongs to a family of transcription factors (reviewed in[21]). The E2F family is currently divided into pro-proliferative (E2F1-E2F3) and anti-proliferative (E2F4-E2F8) members. In quiescent cells, the binding of E2F1-3 to the pocket protein pRb blocks the cell proliferation effects. In the presence of proliferative stimuli, pRB undergoes phosphorylation by cyclin-dependent kinases in complex with their cyclin partners, which in turn allows the release of E2Fs from pRB. Free E2Fs can induce the transcription of many cell cycle-related proteins including cyclin E, which when bound to its cyclin-dependent kinase (cdk) further phosphorylates pRB. This last event increases the amount of free E2Fs which can promote cell cycle progress by inducing the transcription

of many S-phase genes, such as cyclin A and cdk 2.

With regard to the anti-proliferative E2Fs, E2F4 exerts its effect when bound to pRb or one of the other two pocket protein members p107 and p130. In contrast, E2F5 associates preferentially with p130. E2F6-8 seem to down-modulate the expression of E2F-responsive genes, and thus cell proliferation, independently of pocket protein binding.

E2F1 has been implicated in HCC cell growth[22-24] and we have observed that it is also involved in the BZB-induced down-modulation of cell growth in HCC cell lines[20]. With regard to the other E2F members, E2F3-E2F5-E2F8 have been shown to be up-regulated in HCC samples and to play a role in HCC cell growth[25-28].

With the exception of E2F1, little information is available with regard to the possible role of the other E2F family members in the BZB-induced inhibition of proliferation in HCC cell lines. This study investigated the effects of BZB treatment on E2F family members in the HCC cell lines HepG2 and JHH6.

MATERIALS AND METHODS

Cell lines and BZB treatment

The HCC cell lines JHH6 and HepG2 were cultured as reported in[20,24,29]. These cell lines were chosen as they display a different phenotype, hepatocyte-like for HepG2[30] (see also ATCC, catalogue No. HB-8065) and undifferentiated for JHH6[31] (see also Japanese Collection of Research Bioresources (JCRB), catalogue No.: JCRB1030), thus representing suitable *in vitro* models of low and high aggressive forms of HCC, respectively.

BZB was administered as described in[20]; briefly cells were seeded at 3.8×10^3 cells/cm^2 in 6-wells plate, allowed adhering 24 h, cultured for two days in the presence of complete medium and 40 nmol/L BZB. The BZB concentration used was chosen to stay well below the maximal amount/cm^2 recommended dose for *in vivo* application[32]. Moreover, two days of incubation was found to be optimal to study BZB effects on HepG2 and JHH6[20].

Two-color microarray-based gene expression analysis

Two-color microarray-based gene expression analysis was performed according to Agilent Technologies protocol, using an Agilent Scan B (supported with GenePix 4000B scanner and Feature Extraction software, version 9.5.3. – Agilent Technologies, United States).

Briefly, total RNA was extracted using the RNeasy Mini kit (Qiagen GmbH, Germany). The quality, integrity and quantification of total RNA was evaluated by spectrophotometric determination using a Lab-on-Chip-System Bioanalyzer 2100 (Applera Corporation, United States) and a NanoDrop ND-1000 (CelBio, Euroclone, Italy). Fluorescent cRNA (complementary RNA) was created using Agilent's Quick Amp Labeling Kit (Agilent Technologies, United States) with a RNA sample input of 200 ng. For both JHH6 and HepG2, cRNAs were prepared from total RNAs obtained from non BZB-treated cells (NT) and BZB-treated cells (40 nmol/L). The fluorescent dye used for the labelling of NT cRNA was cyanine 3-CTP, (fluorescence emission wavelength of 570 nm) while for BZB-treated cells it was cyanine 5-CTP (fluorescence emission wavelength of 670 nm).

Complementary RNAs were then purified by RNeasy Mini kit (Qiagen S p A., Italy) and quantified using a NanoDrop ND-1000. According to Agilent protocol, for a 4 × 44 K microarray, 825 ng of each cRNAs were used. The Cy-labelled cRNA samples (CY-3 and CY-5 for NT and 40 nmol/L-BZB treated cells, respectively) were mixed and hybridized to a single microarray that was then scanned in a microarray scanner to visualize fluorescence of the two fluorophores. Relative intensities of each fluorophore were used in ratio-based analysis to identify up-regulated and down-regulated genes. Normalization of the data was conducted with RNA-spike ins; *i.e.*, calibrating RNA transcripts. For each mRNA quantified, the Agilent microarray provides a minimum of three and up to ten hybridization oligonucletide probes which matches with different regions of the target mRNA. This means that for any given target mRNA, multiple evaluations were obtained in each of the two independent experiments performed. Microarray analysis were performed 48 h after BZB administration as our previous data[20] indicated this time point to be optimal for BZB effect evaluation.

Quantitative real-time reverse transcription polymerase chain reaction

Part of the total RNAs used for two-color microarray analysis and total RNAs obtained from independent experiments were used to perform quantitative real-time reverse transcription polymerase chain reaction validation. E2F1-8 RT conditions were previously described in[20]; PCR cycles were conducted as follows: pre-denaturation at 95 ℃ for 10 min, 40 cycles of amplification with denaturation at 95 ℃ for 15 s, annealing at proper temperature (Table 1) for 60 s and extension at 72 ℃ for 30 s. A final extension at 72 ℃ for 10-min and a dissociation stage (95 ℃/60 ℃/95 ℃ for 15 s each) was then added. *GAPDH* house-keeping gene was used to normalize data[20].

E2F8 depletion by siRNA

The sequence of the anti E2F8 siRNA (siE2F8, Eurogentec S.A., Belgium) was previously described in[26]. As control (sense 5'-CGUACGCGGAAUACUUCGA-3', antisense: 5'-UCGAAGUAUUCCGCGUACG-3') a siRNA directed against the luciferase gene (siGL2) was analyzed in parallel. Transfections were performed as described in[24] using a weight ratio liposome (Lipofectamin2000 - 1 mg/mL, Invitrogen)/siRNA of 3:1 for three hours at a final siRNA concentration of 220 nmol/L. E2F8 mRNA levels were measured 2 and 4 d after transfection in HepG2 and JHH6, respectively.

Statistical analysis

P values were calculated by the GraphPad InStat tools (GraphPad Software, Inc., La Jolla, CA, United States) using the unpaired *t* test with or without Welch correction and the Mann-Whitney Test, as appropriate. *P* values <

Table 1 Primer sequences performed quantitative real-time reverse transcription polymerase chain reaction

GenBank Number	Protein	Primer pair	TA(℃)	Amplif. Region	Length (bp)
NM_005225	E2F1	(F) 5′-CCAGGAAAAGGTGTGAAATC-3′ (R) 5′-AAGCGCTTGGTGGTCAGATT-3′	62	466-539	74
NM_004091	E2F2	(F) 5′-CAAGTTGTGCGATGCCTGC-3′ (R) 5′-TCCCAATCCCCTCCAGATC-3′	65	645-714	80
NM_001949	E2F3	(F) 5′-AAGTGCCTGACTCAATAGAGAGCC-3′ (R) 5′-AGTCTCTTCTGGACATAAGTAAACCTCA-3′	62	1307-1392	86
NM_001950	E2F4	(F) 5′-GCAGACCCCACAGGTGTTTT-3′ (R) 5′-GCTCCGAGCTCATGCACTCT-3′	62	1081-1162	82
NM_001951	E2F5	(F) 5′-TTGCTTTAATGGTGATACACTTTTGG-3′ (R) 5′-TCTGACCCATTTCTGGAATGG-3′	62	577-659	83
NM_198256	E2F6	(F) 5′-GAAAATGAAAGACTAGCATATGTGACCT-3′ (R) 5′-CTTTAACTGCAATGACGATCTGTTC-3′	62	818-902	85
NM_203394	E2F7	(F) 5′-AGGGATGGAGGTAAATTGTTTAACACT-3′ (R) 5′-TTTCCCCATCTTCAACTGCAA-3′	65	233-318	86
NM_024680	E2F8	(F) 5′-CTGATCTGCGAACAGGATATTAAAAC-3′ (R) 5′-AAAATGAAAAATCTGGAGTTCCTCC-3′	65	399-492	94
NM_002046	GAPDH	(F) 5′-CCCATCACCATCTTCCAGGAG-3′ (R) 5′-CTTCTCCATGGTGGTGAAGACG-3′	62	319-423	105

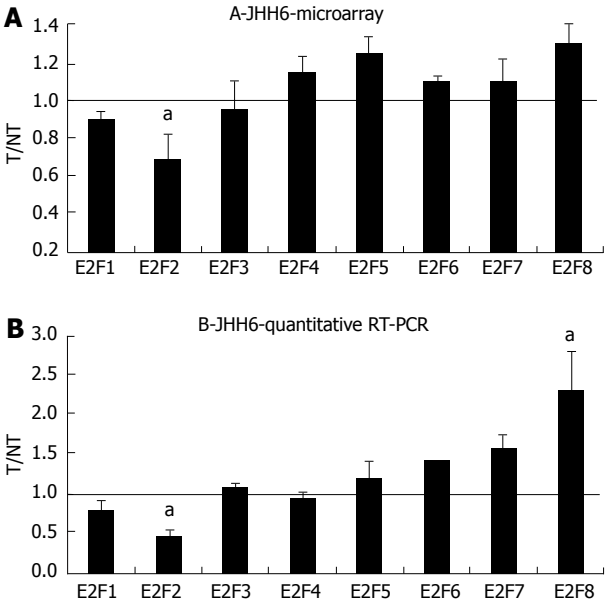

Figure 1 Microarray and quantitative real-time reverse transcription polymerase chain reaction assays in JHH6 following bortezomib treatment. A: The mRNA levels of the indicated E2Fs were evaluated by microarray analysis two days after JHH6 treatment with 40 nmol/L bortezomib. Data are reported as ratio between treated cells (T) and non-treated cells (NT); data are shown as means ± SEM; $^{a}P < 0.05$ *vs* controls; depending on the E2F member the number of evaluation ranged from three up to ten; B: The mRNA levels of the indicated E2Fs were evaluated by quantitative real-time reverse transcription polymerase chain reaction assay; data, normalized to *GAPDH* mRNA, are reported as ratio between T and NT; data are reported as means ± SEM; $^{a}P < 0.05$ *vs* controls, *n* = 5. RT-PCR: Reverse transcription polymerase chain reaction.

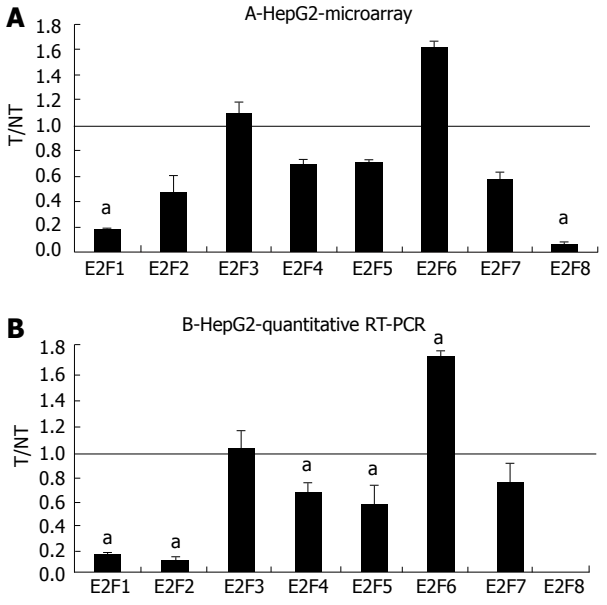

Figure 2 Microarray and quantitative real-time reverse transcription polymerase chain reaction assays in HepG2 following bortezomib treatment. A: The mRNA levels of the indicated E2Fs were evaluated by microarray analysis two days after JHH6 treatment with 40 nmol/L bortezomib. Data are reported as ratio between treated cells (T) and non-treated cells (NT); data are shown as means ± SEM $^{a}P < 0.05$ *vs* controls; depending on the E2F member the number of evaluation ranged from three up to ten. B: The mRNA levels of the indicated E2Fs were evaluated by quantitative real-time reverse transcription polymerase chain assay; data, normalized to *GAPDH* mRNA, are shown as ratio between T and NT; data are reported as means ± SEM; $^{a}P < 0.05$ *vs* controls, *n* = 5. RT-PCR: Reverse transcription polymerase chain reaction.

0.05 were considered to be statistically significant.

RESULTS

Effects of BZB on E2F family members

We have evidence that E2F1 is dramatically down-regulated upon BZB treatment in the differentiated HCC cell line HepG2[20]. In contrast, in the undifferentiated HCC cell line JHH6 E2F1 down-regulation is only modest. This observation prompted us to verify whether other E2F members are involved in the BZB induced inhibition of proliferation in JHH6. Microarray analysis performed in JHH6 (Figure 1A) following 48 h treatment by BZB at 40 nmol/L, revealed a decrease in the expression of the pro-proliferative E2F member E2F2 and confirmed the modest decrease of E2F1 we previously observed[20]. With regard to the anti-proliferative E2F members, we observed a tendency of BTZ treatment to increase mRNA levels of most E2F members. Quantitative reverse transcription polymerase chain reaction (RT-PCR) data (Figure 1B) confirmed a significant decrease and increase in E2F2 and E2F8 expression.

Compared to the undifferentiated JHH6, the hepatocyte-like HCC cell line HepG2, displayed a more marked effect of BZB on the differential expression of E2F members, as shown by microarray analysis and confirmed by quantitative RT-PCR (Figure 2). In the HepG2 cell line we detected a stronger down-regulation of the expression of the pro-proliferative E2F members E2F1 and E2F2 (Figure 2B) and of the anti-proliferative member E2F8,

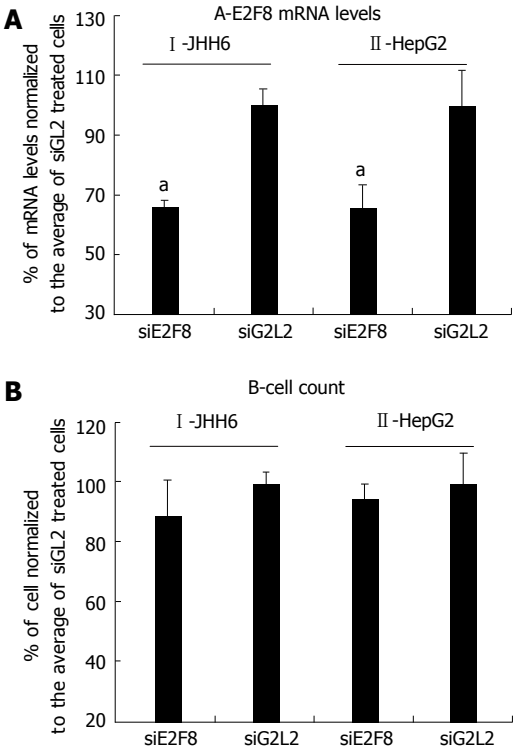

Figure 3 Effects of E2F8 depletion by siRNA in HepG2 and JHH6. A: Four and 2 d after siRNA administration, the reductions of E2F8 mRNA in JHH6 and HepG2, respectively, are reported; the data, normalized to GAPDH levels, are shown as mean ± SEM; $^{a}P < 0.05$ *vs* controls, $n = 6$; B: the effects of siE2F8 on cell number 4 and 2 d after siRNA administration in JHH6 and HepG2, respectively, are reported; the data, normalized to siGL2 treated cells, are shown as mean ± SEM, $n = 6$. siE2F8: siRNA against E2F8 mRNA; siGL2: Control siRNA against the luciferase mRNA.

with E2F4 and E2F5 being less affected. As with JHH6, BZB treatment of HepG2 cells induced the significant up-regulation of the expression of an anti-proliferative E2F member which, in this case, it was E2F6.

The marked down-regulation of the expression of the anti-proliferative E2F8 transcription factor in the HepG2 cell line would suggest a pro-proliferative effect of BZB rather than an anti-proliferative effect. However, recently[26] it has been observed that E2F8 depletion by siRNA resulted in the down-regulation of cell proliferation in HCC cell lines. Thus, we verified whether this could have been the case in the HepG2 cell line. Using the siRNA previously proposed[26], we observed that whereas E2F8 siRNA targeting could significantly reduce E2F8 mRNA levels (Figure 3A), no major effects on cell number (Figure 3B) were detected 2 d after a single siRNA transfection. Notably, the siRNA mediated depletion of E2F8 in JHH6 also did not result in a significant decrease in cell number (Figure 3A Ⅰ and B Ⅰ). In this last case cell counting was performed four days after siRNA transfection as at this time point the siRNA effect on E2F8 mRNA level was maximum.

Effects of BZB on other cell cycle gene products

The more marked effect of BZB on the differential expression of E2F members in the HepG2 cell line compared to JHH6 prompted us to verify whether this behaviour was restricted to the E2F family or it was a more general feature. For this reason we compared, by microarray analysis, the effects on the differential expression of cyclins (Figure 4) and cdks (data not shown). In the case of cyclin E, cyclin D, cyclin A and cdk2 the microarray data have been previously validated by quantitative RT-PCR[20]. From the analysis of BZB effects on the expression levels of cyclin and *cdk* gene products, little change was observed with the undifferentiated JHH6 compared to the more differentiated HepG2 cell line. Notably, this behaviour was not restricted to cell cycle genes, and was also seen with the expression of epithelial-mesenchymal transition (EMT) genes; these genes allow the cancer cells to gain migratory and invasive properties, thus resulting in the possibility of metastasis. In JHH6, the impact of BZB on EMT gene expression was definitively less pronounced than in the HepG2 cell line (Figure 5). The reduced BZB impact on gene expression in JHH6 compared to HepG2 cells is also shown by the fact that BZB treatment of HepG2 cells modified the signal coming from about 12000 gene probes, whereas in JHH6 this number was of about 2000.

DISCUSSION

Several studies suggest that BZB is a potent 26S proteasome inhibitor with the potential to be of therapeutic value for HCC treatment. We have previously observed[20] that BZB can effectively inhibit the growth of the differentiated HCC cell line HepG2 and, to a lesser extent, also that of the undifferentiated HCC cell line JHH6. We can exclude that this difference arises from a reduced BZB-mediated inhibition of the 26S proteasome in JHH6 cells compared to HepG2 as a similar reduction of proteasome activity was previously shown in these two cell lines[20]. It follows that also the effects on gene expression reported in this work unlikely depend on a differential inhibition of the 26S proteasome by BZB in the two cell lines.

The role of E2F family members in HCC has been mainly demonstrated for E2F1[20,22] whose up-regulation, considered an unfavorable prognostic factor[23], has been shown in HCC[33]. The other E2F members studied in the literature are E2F3, E2F5 and E2F8 for which indication of the up-regulation in HCC samples and implication in HCC cell growth were reported[25-28]. The lack of information about the other E2F family members in HCC development in general and in relation to BZB effects, prompted us to start a novel investigation in this field.

In addition to confirming in JHH6 our previous data of a modest effect on E2F1 mRNA by BZB[20], this study suggests that the differential expression of other E2Fs contributes to BZB's action in JHH6. Interestingly, the down-regulation of the pro-proliferative E2F2 (Figure 1B) and the up-regulation of the anti-proliferative E2F8 suggest a net anti-proliferative effect of BZB. The differential expression of these two E2Fs is not dramatic. It

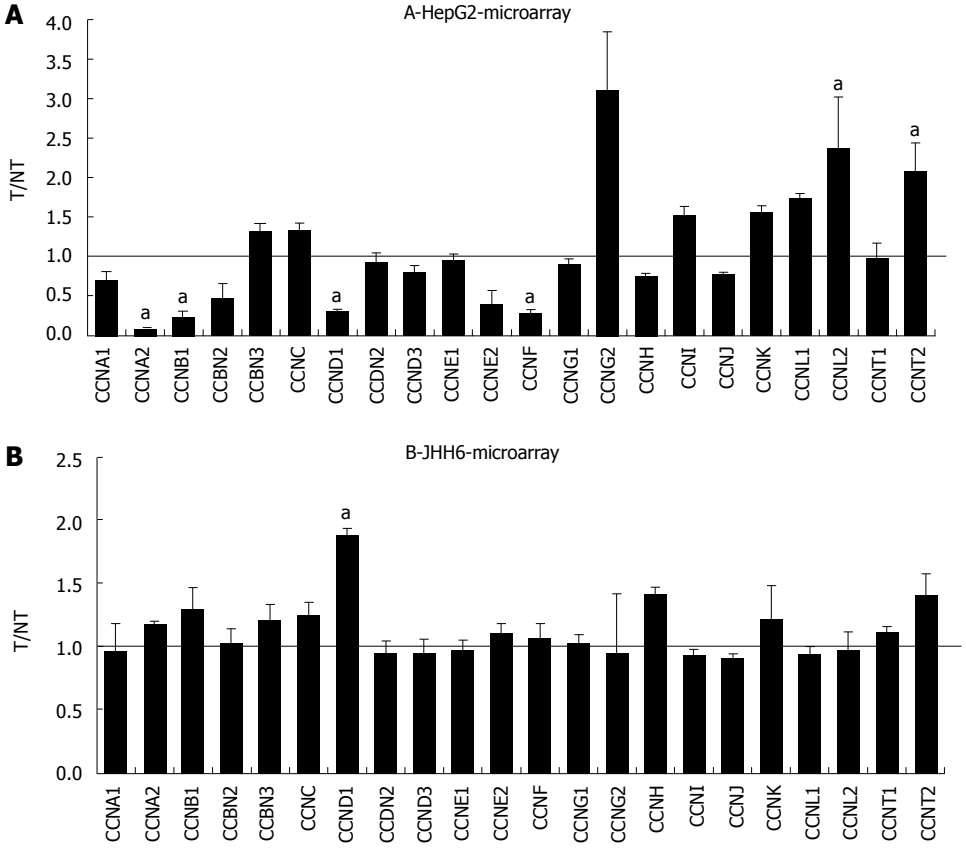

Figure 4 Microarray assay in HepG2 and JHH6 following Bortezomib treatment. The mRNA levels of the indicated cyclins were evaluated by microarray analysis two days after HepG2 (A) or JHH6 (B) treatment by 40 nmol/L Bortezomib. Data are reported as ratio between treated cells (T) and non-treated cells (NT); data are shown as means ± SEM; $^{a}P < 0.05$ *vs* controls; depending on the cyclin member, the number of microarray evaluation ranged from three up to ten.

is thus possible that BZB's anti-proliferative effect stems from the sum of multiple "minor" anti-proliferative signals. In this sense the slight increase of the other anti-proliferative E2F members E2F5, E2F6 and E2F7 may contribute to the global anti-proliferative effect. Future functional investigations will further clarify the contribution of each of the differentially expressed E2F members in the down-regulation of JHH6 proliferation.

In the differentiated HCC cell line HepG2, BZB effects on the expression of the pro-proliferative E2Fs, although qualitatively similar to JHH6 (E2F1 and E2F2 down-regulation with E2F3 substantially unaffected; Figure 2B), are quantitatively more pronounced. The reasons for this quantitative difference deserve additional investigation. These observations are in line with preliminary studies conducted in another HCC cell line HuH7 (Dapas *et al*, unpublished results), which display an intermediate differentiation grade, showing an effect on E2Fs intermediate between JHH6 and HepG2.

In the HepG2 cell line, BZB effects on the anti-proliferative E2Fs is, in contrast, substantially different from that observed in JHH6 (compare Figure 2B with 1B), characterized by the up-regulation of E2F6 and by the marked down-regulation of E2F8. Particularly intriguing is the dramatic down-regulation of E2F8. Despite being known as an anti-proliferative E2F[21], E2F8 has been recently suggested by Deng *et al*[26] to promote HCC cell proliferation. Our data (Figure 3) indicate that E2F8 depletion does not seem to have a major effect on HepG2 growth; this event is not limited to HepG2 cells as the same occurs with JHH6 (Figure 3). These contrasting data with Deng *et al*[26] may firstly depend on the fact that we have used different HCC cell lines (HepG2/JHH6 *vs* YY-8103/Focus cells). In addition, we measured cell growth at day two and four in HepG2 and JHH6, respectively, following E2F8 depletion; in contrast, Deng *et al*[26] prolonged the analysis up to day 6, a time point at which the anti-proliferative effect was most evident. We limited the analysis at day two/four in HepG2 and JHH6, respectively, as at these time points the effect was maximal with a single siE2F8 administration. Additionally, the overgrowth of control cells (non-treated cells and control siRNA-treated cells) at longer time points made cell growth evaluation (by cell counting) cumbersome. Together, our observations suggest that, at least in the HCC cell lines considered and under our experimental conditions, E2F8 does not seem to be a major promoter of cell proliferation.

The decrease of most of the anti-proliferative E2F members in the HepG2 cell line treated by BZB would favor the concept of a pro-proliferative effect. However, it should be considered that the prediction of the net effect of the E2Fs deregulation is not so straightforward. This is due to the redundancies of E2F functions[21] which allow one member to take the part of another one.

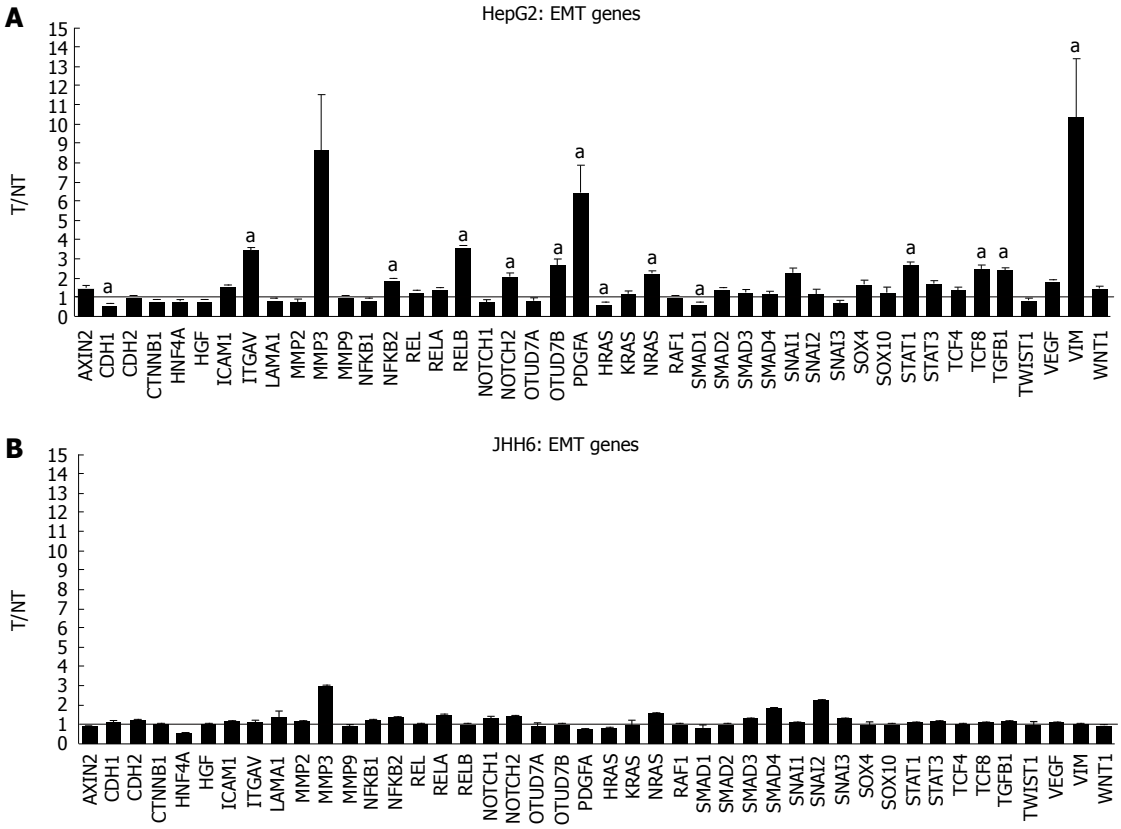

Figure 5 Microarray assays in HepG2 and JHH6 following bortezomib treatment. The mRNA levels of the indicated epithelial-mesenchymal transition (EMT) genes were evaluated by microarray analysis two days after HepG2 (A) or JHH6 (B) treatment by 40 nmol/L Bortezomib. Data are reported as ratio between treated cells (T) and non-treated cells (NT); data are shown as means ± SEM; $^{a}P < 0.05$ *vs* controls; depending on the EMT member, the number of microarray evaluation ranged from three up to ten.

It is thus possible that E2F6 takes the place of the down-regulated E2F4, E2F5 and E2F8, favoring the anti-proliferative effect. This, together with the down-regulation of E2F1 and E2F2, may explain the net anti-proliferative effect.

The substantial reduced quantitative impact of BZB on the expression of E2F family members in JHH6 when compared to HepG2 occurs also for gene products related and unrelated to E2Fs, such as cyclins/cdks and EMT genes, respectively. As remarked above, the differences in BZB effects in JHH6 and HepG2 cells do not seem to be related to proteasome-dependent mechanisms, but possibly to proteasome-independent mechanisms. Despite the reasons for this behavior, these findings are in line with the more contained impact of BZB in the undifferentiated JHH6 when compared to the more differentiated HepG2 cell line. This observation, together with our previous data[20], strongly suggests that not all the HCC types may respond, from the quantitative point of view, in a similar manner to BZB treatment. In this regard, it is interesting to note the negligible effects of BZB on the expression of EMT genes in JHH6. As these genes are involved with metastasis, it is possible that BZB treatment does not effectively prevent metastasis, at least in some HCC cell phenotypes.

In summary, we reported for the first time data indicating that in the HCC cell lines tested, BZB effects on the expression of E2F family members are not limited to E2F1 but are extended to other members. In particular, in both JHH6 and HepG2 the expression of the pro-proliferative E2F2 is down-regulated. Specific to JHH6 is the up-regulation of the expression of the anti-proliferative E2F8, while in the HepG2 cell line it is the up-regulation of the anti-proliferative E2F6 and the marked repression of E2F8 expression. Together these data expand our knowledge on the molecular basis of BZB action in inhibiting the proliferation of HCC cells, strengthening the rationale for its future use in HCC-affected patients.

COMMENTS

Background

In the last decade the drug bortezomib (BZB) has been studied as a possible novel therapeutic treatment for hepatocellular carcinoma (HCC). BZB is a boronic acid dipeptide derivative able to inhibit the 26S proteasome.

Research frontiers

The other E2F members studied in the literature are E2F3, E2F5 and E2F8 for which indication of the up-regulation in HCC samples and implication in HCC cell growth were reported.

Innovations and breakthroughs

The differential expression of E2Fs and related genes induced by BZB in diverse HCC cell phenotypes contribute to bortezomib's mechanism of action in hepatocellular carcinoma.

Applications

The authors reported for the first time data indicating that in the HCC cell lines

tested, BZB effects on the expression of E2F family members are not limited to E2F1 but are extended to other members.

Peer review

The article is an original research paper focusing on the proteasome inhibitor BZB effects on E2Fs transcription factors and related genes in HCC. The study is well structured, the subject is actual and interesting, providing a rationale for performing the research.

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BRIEF ARTICLE

Systematic review: Laparoscopic fundoplication for gastroesophageal reflux disease in partial responders to proton pump inhibitors

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Abstract

AIM: To assess laparoscopic fundoplication (LF) in partial responders to proton pump inhibitors (PPIs) for gastroesophageal reflux disease (GERD).

METHODS: We systematically searched PubMed and Embase (1966-Dec 2011) for articles reporting data on LF efficacy in partial responders. Due to a lack of randomized controlled trials, observational studies were included. Of 558 articles screened, 17 were eligible for inclusion. Prevalence data for individual symptoms were collated across studies according to mutually compatible time points (before and/or after LF). Where suitable, prevalence data were presented as percentage of patients reporting symptoms of any frequency or severity.

RESULTS: Due to a lack of standardized reporting of symptoms, the proportion of patients experiencing symptoms was recorded across studies where possible. After LF, the proportion of partial responders with heartburn was reduced from 93.1% (5 studies) to 3.8% (5 studies), with similar results observed for regurgitation [from 78.4% (4 studies) to 1.9% (4 studies)]. However, 10 years after LF, 35.8% (2 studies) of partial responders reported heartburn and 29.1% (1 study) reported regurgitation. The proportion using acid-suppressive medication also increased, from 8.8% (4 studies) in the year after LF to 18.2% (2 studies) at 10 years. In the only study comparing partial responders to PPI therapy with complete responders, higher symptom scores and more frequent acid-suppressive medication use were seen in partial responders after LF.

CONCLUSION: GERD symptoms improve after LF, but subsequently recur, and acid-suppressive medication use increases. LF may be less effective in partial responders than in complete responders.

Key words: Laparoscopic fundoplication; Gastroesophageal reflux disease; Partial response, Proton pump inhibitors; Systematic review

Core tip: There are no high-level evidence to support the use of laparoscopic fundoplication (LF) in partial responders to proton pump inhibitor (PPI) therapy. The evidence that does exist suggests LF improves symptom control in these patients, but symptoms recur over time. There are limited data to suggest that LF is not as efficacious in partial responders as in those with an adequate response to PPI therapy.

Lundell L, Bell M, Ruth M. Systematic review: Laparoscopic fundoplication for gastroesophageal reflux disease in partial responders to proton pump inhibitors. *World J Gastroenterol*

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INTRODUCTION

Gastroesophageal reflux disease (GERD) affects approximately 10%-20% of adults in Western countries, with cardinal symptoms of heartburn and regurgitation[1]. Current treatment options include pharmacological and surgical approaches. The most common pharmacological therapy is acid-suppression with proton pump inhibitors (PPIs) (or, less commonly, histamine-2 receptor antagonists)[2]. GERD symptoms can also be treated surgically by the fundoplication technique, which involves wrapping the gastric fundus partly or completely around the lower end of the esophagus. More recently, the introduction of laparoscopic techniques has reduced perioperative complications and facilitated postoperative recovery, without compromising the level of GERD control. These improvements could increase the likelihood of patients being referred for this procedure.

The clinical effectiveness of laparoscopic fundoplication (LF) has been extensively validated[3], but the critical question remains of how to select those patients with chronic GERD who will benefit most from such procedures. Most studies in the surgical literature have included only patients who respond adequately to PPIs (referred to as "complete responders" from this point onwards), but it is increasingly evident that a substantial proportion of patients experience only partial or no relief from reflux symptoms, even after optimized PPI treatment[4,5]. These patients are often referred to as "partial responders" or "non-responders", depending on the degree of symptom alleviation[6].

Partial responders to PPI treatment are commonly referred for LF, even though evidence for the effectiveness of the procedure in these patients has not been systematically assessed. The aim of this study was therefore to systematically review data on the effectiveness of LF in partial responders to PPI treatment and thereby offer a scientific platform for clinical decision-making in patients with complex GERD.

MATERIALS AND METHODS

Search strategy

Targeted literature searches were conducted in PubMed and Embase from 1966 until December 2011. Reviews, studies not conducted in adult humans and studies not published in English were excluded using search engine filters. The remaining studies were screened based on titles and abstracts, and full articles were reviewed when their relevance was unclear from the abstract.

Study selection

Study eligibility was assessed by all three authors. The primary targets were randomized controlled trials (RCTs) comparing LF with PPI maintenance therapy in partial responders. However, no such trials were identified and the selection criteria were therefore expanded to include any prospective studies assessing LF in partial responders, preferably including baseline data obtained before LF while patients were on PPI therapy. The search methodology used to identify relevant articles is summarized in Figure 1.

Data abstraction

Data were abstracted by a single author (Bell M) and reviewed by the co-authors (Lundell L and Ruth M). Multiple methods of reporting symptoms obscure comparisons among studies. To minimize the impact of this variation, the authors focused (where possible) on collecting data on the prevalence of individual symptoms of any frequency and/or severity. These data were collated across studies according to mutually compatible time points (before and/or after LF). Where suitable, prevalence data were presented as percentage of patients reporting symptoms of any frequency or severity. Any other data providing insight into the effectiveness of LF, such as physiological or quality of life (QoL) measurements, were also collected. Nissen, Nissen-Rossetti, robot-assisted Nissen and Toupet, as well as other partial fundoplications, were all included under the broader definition of LF. Resting lower esophageal sphincter pressure (LESP) values reported in kPa were converted to mmHg, using standard conversion criteria stated by the International Bureau of Weights and Measures (1 mmHg = 0.133322 Pa).

RESULTS

Study characteristics

Of 558 articles screened, 17 (reporting data from 13 trials) were deemed eligible for inclusion, with sample sizes of partial responders undergoing LF ranging from 10 to 1340; study characteristics are summarized in Table 1[7-23]. A PRISMA diagram outlining search strategy, study elimination and study selection is presented in Figure 1. Of the 17 articles, five (three trials) were conducted in the Netherlands, three (two trials) in the United Kingdom, two each in Austria and Italy, and one each in Canada, France, Belgium, Switzerland and Greece. The prevalence of individual symptoms (any frequency or severity) was reported by eleven studies (heartburn[7-10,16,17,19-21], regurgitation[7-10,16,17,20,21] and dysphagia[7-10,15-17,19-21,23]). Physiological reflux measures were presented by thirteen studies (LESP[7-10,16,18,20-23], esophageal acid exposure[7-9,11,12,18,19,21-23] and endoscopic evaluation of reflux esophagitis[7-10,18,21,22]). QoL measures were reported by nine studies [Gastrointestinal Quality of Life Index (GIQLI)[10,16,17], GERD Health-Related Quality of Life (GERD-HRQL)[16,21,22] or general QoL visual analogue scales(VASs)[7-9,18,22]]. Other measures indicative of surgical effectiveness were provided by eleven studies (patient satisfaction with the outcome of LF[7-10,15,18-20], postoperative use of acid-suppressive

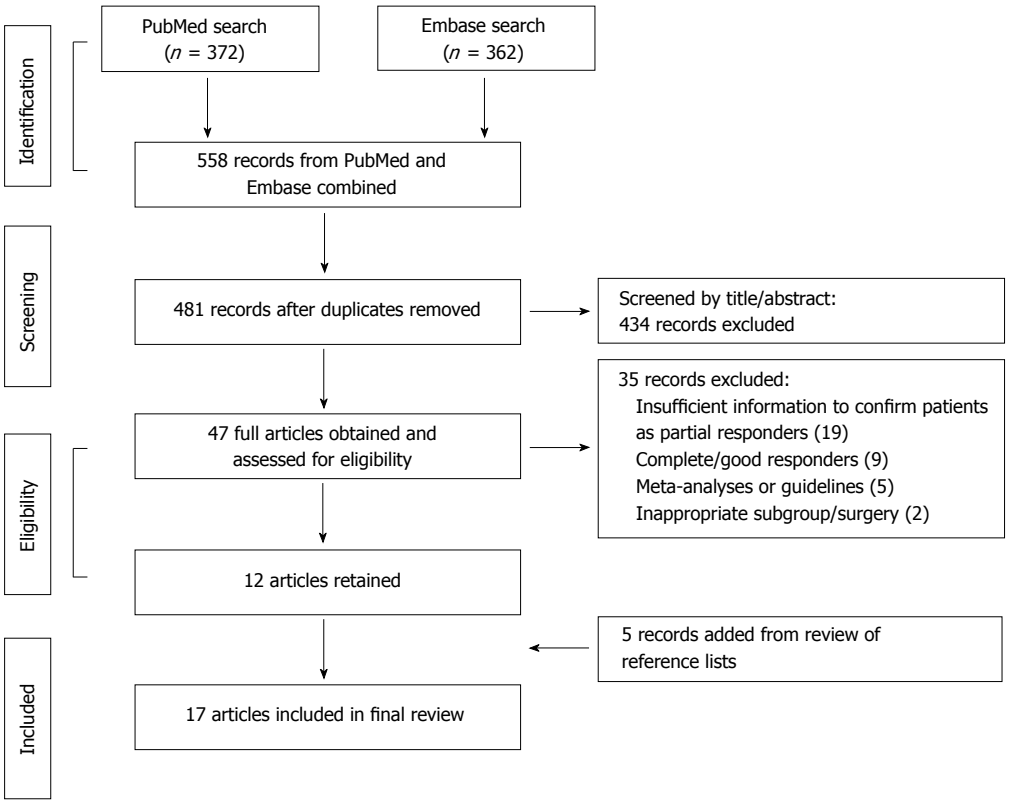

Figure 1 Search strategy.

medication[7-9,11,15-20], intraoperative conversion to open fundoplication[10,15,17,18,20] and surgical reintervention rates after LF[7-10,15-20]). The definitions of partial response varied substantially across studies (Table 1).

Impact of laparoscopic fundoplication on symptoms of GERD in partial responders

Heartburn: Data on the prevalence of heartburn (any frequency or severity) after LF were reported in seven trials[7-10,16,17,19-21]. Pooled data from these studies, some of which did not report baseline data (heartburn prevalence before LF in patients taking a PPI)[16,19], indicate that the proportion of patients experiencing heartburn decreased substantially in the year after LF but recurred over time, with 35.8% (53/148) of patients across two studies reporting heartburn after 10 years (Figure 2A)[9,17].

Of the five trials reporting prevalence data before (during PPI treatment) and after LF, two reported complete remission of heartburn at 1 year after LF, from 60% (18/30) and 91.4% (32/35) before LF to 0% (0/30 and 0/28, respectively) after[20,21]. Another study reported that heartburn prevalence decreased from 95.5% (148/155) before LF to 0% (0/155) 1 year after and 2.6% (1/39) 5 years after[10]. In the two trials reporting the longest follow-up, heartburn prevalence was reduced from 93% (53/57) and 100% (100/100) before LF to 40.5% (32/79) and 30.4% (21/69) 10 years after, respectively[7,9,17].

Regurgitation: Data on the prevalence of regurgitation (any frequency or severity) after LF were reported in six trials[7-10, 16,17,20,21]. Pooled data from these studies, some of which did not report baseline data (regurgitation prevalence before LF in patients taking a PPI)[7,16], indicate that the proportion of patients experiencing regurgitation decreased substantially in the year after LF but recurred over time, with 29.1% (23/79) of patients reporting regurgitation 10 years after LF in the only study reporting data over this period (Figure 2B)[9].

Of the four trials reporting prevalence data before (during PPI treatment) and after LF, two reported a decrease 1 year after LF, from 71.4% (25/35) and 93.3% (28/30) before LF to 3.6% (1/28) and 13.3% (4/30) after, respectively[20,21]. In the two trials reporting the longest follow-up, one reported that regurgitation prevalence was reduced from 54% (54/100) before LF to 17.4% (15/86) 5 years after, and the other reported that regurgitation prevalence was reduced from 92.9% (144/155) before LF to 0% (0/155) 1 year after and 5.1% (2/39) 5 years after[10,17].

Dysphagia: Data on the prevalence of dysphagia (any frequency or severity) after LF were reported in nine trials[7-10,15-17,19-21,23]. Pooled data from these studies, some of which did not report baseline data (dysphagia prevalence before LF in patients taking a PPI)[7,15,16,19,23], indicate that the proportion of patients experiencing dysphagia increased slightly in the 6 mo after LF, before decreasing substantially after 1 year (Figure 2C). The response appears to be biphasic, with the prevalence remaining low at 5 years[10,15,17,19], but increasing in the two trials with a 10-year follow-up period[9,17].

Table 1 Details of selected studies

Ref.	Study design	Country	Definition of partial response	Age range (yr)	*n*
Bais *et al*[7] Draaisma *et al*[8] Broeders *et al*[9]	RCT 10-yr follow-up of LNF *vs* CNF	Netherlands	Bais *et al*: "…in patients with symptoms of GORD, insufficiently reacting to at least 40 mg of omeprazole daily, persisting oesophagitis, and pathological acid exposure, surgical treatment was proposed"… Broeders *et al*: "177 patients were included in a multi-center RCT to undergo Nissen fundoplication for PPI-refractory GERD"	17-79	79
Granderath *et al*[10]	Prospective 5-yr follow-up of LTF	Austria	"All patients had a long history of GERD symptoms (mean 7.1 years; range 6 mo to more than 10 yr) and had been receiving medical treatment with PPI for a mean period of 18.4 ± 6.8 mo (20-60 mg omeprazole daily)"	29-74	155
Anvari *et al*[11]	Prospective 5-yr follow-up of LF (one cohort of "poor responders")	Canada	"Inadequate response (< 70% relief on a visual analogue scale, defined by the patient's subjective impression) to PPIs titrated to a dose of 120 mg/d in 445 patients"	Not specified	445
Jenkinson *et al*[12]	Prospective 6-8-wk follow-up after LF	United Kingdom	"All patients were on long term acid-suppression therapy (at least 4 mo) with PPIs and were either symptomatic or dissatisfied with the treatment"	20-78	70
Mahon *et al*[13] Mehta *et al*[14]	RCT 7-yr follow-up of LF *vs* PPI in GERD (PR subset)	United Kingdom	Mehta *et al*: "patients with symptoms of GERD for at least 6 mo" with "3 mo minimum of PPI maintenance therapy"..."After 12 mo, those who had been randomized to PPI were offered the opportunity to have surgery"	26-69	54
Pessaux *et al*[15]	Retrospective 5-yr follow-up of LF	France	"The indications for surgery were intractable or recurrent symptoms due to GERD after an adequate trial (minimum of 3 mo) of conservative treatment that consisted of proton pump inhibitors (n = 1234; 92.1%)"	18-86	1340
Ciovica *et al*[16]	Prospective 12-mo follow-up of LF	Austria	"Antireflux surgery was indicated and performed in patients with persistent or recurrent GERD symptoms and/or complications despite maximal conservative treatment, and in patients preferring surgery to medical treatment"	16-81	351
Dallemagne *et al*[17]	Prospective 5- and 10-yr follow-up of LF questionnaires	Belgium	"All patients were taking PPIs for acid suppression" … "For all patients, the primary symptoms indicating surgery were heartburn, associated with regurgitation in 54% and dysphagia in 8% of the patients"	10-78	100
Draaisma *et al*[18]	RCT 6-mo follow-up of robot-assisted *vs* standard laparoscopy LF	Netherlands	"Surgical treatment was proposed for patients with GORD insufficiently reacting to proton pump inhibitors..."	20-74	50
Zehetner *et al*[19]	Prospective 5-yr follow-up of LTF	Switzerland	All patients diagnosed with GERD according to SAGES criteria; "98% received a pre-operative medical treatment for > 3 mo with PPI/H_2-blockers and/or prokinetic"	27-81	100
Antoniou *et al*[20]	Prospective 1-yr follow-up of LNF	Greece	"Continuous doses of PPIs for a minimum period of 6 mo incurred no or only partial relief of their symptoms. More specifically, refractory GERD symptoms to a standard dose of PPIs (omeprazole 20 mg/d) for at least 4 mo were followed by a therapeutic trial with a double dose of PPIs (omeprazole 40 mg/d) for 8-10 wk. Patients not responding to the high-dose PPI treatment were included in the study"	17-65	32
Brillantino *et al*[21]	Prospective 1-yr follow-up of LNF	Italy	"…patients referred for symptomatic gastroesophageal reflux poorly responsive to standard dose PPI therapy…" "...persisting heartburn or regurgitation during treatment for at least 3 mo with omeprazole at 40 mg or lansoprazole at 30 mg/d…"	18-70	35
Broeders *et al*[22]	Prospective 1-yr follow-up of LNF	Netherlands	"Thirty-one patients with PPI-refractory GORD with pathological acid exposure on pH monitoring…"	26-67	31
Frazzoni *et al*[23]	Prospective 3-mo follow-up of EsophyX *vs* LNF	Italy	"...adult patients referred to our centre because of troublesome heartburn/regurgitation persisting despite at least 4-wk high-dose PPI therapy"	"Adults"	10
				Total:	2852

CNF: Conventional Nissen fundoplication; GERD: Gastroesophageal reflux disease; GORD: Gastro-oesophageal reflux disease; H_2: Histamine-2 receptor; LF: Laparoscopic fundoplication; LNF: Laparoscopic Nissen fundoplication; LTF: Laparoscopic Toupet fundoplication; NA: Not available; PPI: Proton pump inhibitor; PR: Partial responder; RCT: Randomized controlled trial; SAGES: Society of American Gastrointestinal and Endoscopic Surgeons.

Of the three studies reporting prevalence data before (during PPI treatment) and after LF, two showed an improvement in symptoms 1 year after LF[10,20], from 43.9% (68/155) and 26.7% (8/30) before LF, to 2.6% (4/155) and 6.7% (2/30) after, respectively; in the former study, the decrease in dysphagia prevalence after LF was well maintained at 5-year follow-up with 5.1% (2/39) of patients reporting dysphagia[18]. However, the remaining study, with the longest follow-up period, showed an increase in the number of patients reporting dysphagia,

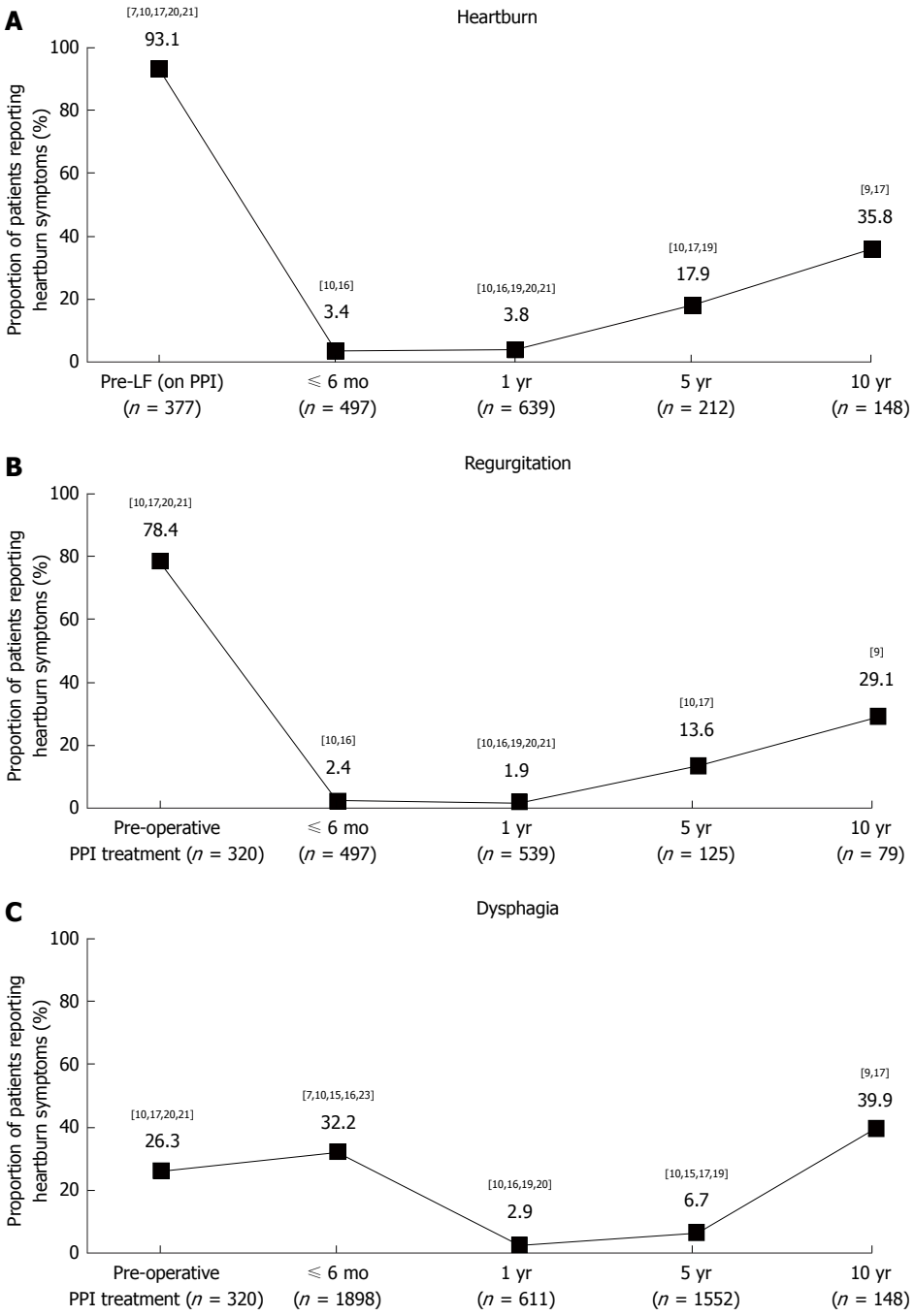

Figure 2 Proportion of patients reporting heartburn, regurgitation and dysphagia symptoms during preoperative proton pump inhibitor treatment and at follow-up after laparoscopic fundoplication. Superscript numbers indicate individual studies. A: Heartburn; B: Regurgitation; C: Dysphagia.

from 8% (8/100) before LF to 37.2% (32/86) 5 years after and 23.2% (16/69) 10 years after[17].

Impact of laparoscopic fundoplication on acid reflux, lower esophageal sphincter pressure and reflux esophagitis in partial responders

Ambulatory 24-h pH measurements: In five trials, esophageal acid exposure (proportion of time with esophageal pH < 4 during ambulatory 24-h pH measurements) was assessed before LF in patients who had not taken a PPI for at least 3 d, and again 0.25-1 years after LF[7-9,12,18,19,22]. Across these studies, esophageal acid exposure was reduced by 85.5%-97.0%, from 8.5%-17.8% (n = 753) before LF to 0.3%-1.8% (n = 242) after. In the only one of these trials reporting such data 5 years after LF[7,8], the 85.5% (n = 57) reduction in esophageal acid exposure observed 1 year after LF was largely maintained [82.3% (n = 48)].

In three studies, esophageal acid exposure was measured before LF in patients who were still taking a PPI, and repeated 0.25-1 year after LF (n = 115)[12,21,23]. Across these studies, esophageal acid exposure was reduced by 58.6%-100.0%, from 0.5%-4.9% (n = 115) before LF to 0.5%-2.0% (n = 108) after. Of these, one study reported

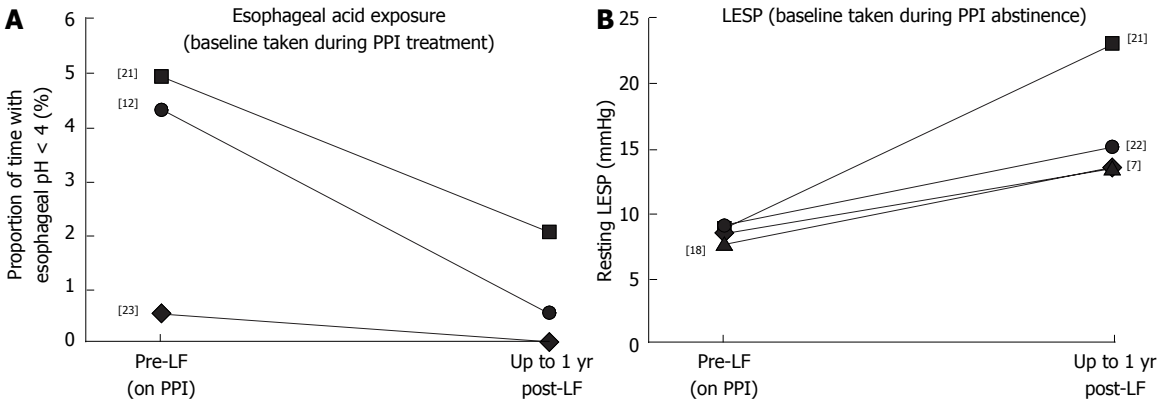

Figure 3 Effects of laparoscopic fundoplication on esophageal acid exposure (assessed by ambulatory 24-h H measurement) and resting lower esophageal sphincter pressure (assessed by esophageal manometry). Data are shown for before and after laparoscopic fundoplication (LF) (follow-up period: 0.25-1 year). A: Esophageal acid exposure [baseline taken during proton pump inhibitors (PPIs) treatment]; B: Lower esophageal sphincter pressure (LESP) (baseline taken during PPI abstinence).

Table 2 Endoscopy-proven esophagitis scores (Los Angeles classification[24])

	Pre-LF						Follow-up						
	None	A	B	C	D	*n*		None	A	B	C	D	*n*
Bais *et al*[7]	29	17	7	1	1	57[1]	No follow-up data presented						
Granderath *et al*[10]	12	41	55	24	28	160	No follow-up data presented						
Draaisma *et al*[18]	14	11	13	3	3	44	6 mo	43	1	2			46
Brillantino *et al*[21]	10	21 (A or B)		4 (C or D)		35	12 mo	34	1	0	0	0	35
Broeders *et al*[22]	16	8	4	2	1	31	6 mo	25	3	2	0	0	30

[1]Endoscopy results could not be determined in two patients. LF: Laparoscopic fundoplication.

reductions in esophageal acid exposure of 100%, but data were available for only 10 patients[23]. In the other two studies, esophageal acid exposure was reduced by 58.6% (n = 35)[21] and 88.4% (n = 70)[12] after LF. These changes are summarized in Figure 3A.

In the only study to compare esophageal acid exposure after LF with pre-LF measurements obtained on and off PPI therapy, esophageal acid exposure was lower in patients after LF (median: 0.5%) than in patients before LF, whether they were on or off PPIs [4.3% and 9.5%, respectively (n = 70)] [12].

Lower esophageal sphincter pressure: In four trials, resting LESP was assessed before LF in patients who had not taken a PPI for at least 3 d, and again 0.25-1 years after LF. Across these studies, resting LESP increased by 62.7%-164.4%, from 7.5-9.0 mmHg (n = 164) before LF to 13.5-23.0 mmHg (n = 157) after[7-9,18,21,22]. These changes are summarized in Figure 3B. In one of these trials, the follow-up period was extended to 5 years (n = 48)[7,8]. The 62.7% increase in LESP seen in this trial between baseline (8.3 mmHg) and 3 mo after LF (13.5 mmHg) was largely maintained after 5 years (12.8 mmHg, or a 54.2% increase relative to baseline). Only one study presented baseline data during PPI therapy compared with after LF; however, the values presented in this study (median value of 18 mmHg during PPI therapy before LF *vs* 21 mmHg after) are much higher than those reported by other studies, and furthermore, this study only included 10 patients[23].

Endoscopic evaluation of reflux esophagitis: Across the five trials presenting LA classification data (Table 2)[24], 74.6% (244/327) of participants were found to have some degree of reflux esophagitis before LF, although no study clarified whether pre-LF endoscopic assessments were taken during PPI treatment[7-10,18,21,22]. Grade C or D reflux esophagitis was found in 20.5% (67/327) of patients. Of the three studies reporting LA classification data both before and 6-12 mo after LF, all revealed substantial reductions in the proportion of patients with endoscopy-proven reflux esophagitis (range across studies at baseline: 48.4%-71.4%; range after LF: 2.9%-16.7%)[18,21,22]. Collectively, the proportion of patients presenting with reflux esophagitis was reduced from 63.6% (70/110) before LF to 8.1% (9/111) after; no patients were reported to have grade C or D reflux esophagitis after LF.

Impact of laparoscopic fundoplication on quality of life measurements in partial responders

Only four trials compared QoL before LF while patients were taking a PPI with that after LF[7-9,16,21,22]. Of these, three found that GERD-HRQL scores improved (decreased) 1 year after LF [12-20.1 to 1-3.5 (n = 417)][16,21,22], two reported improvements (increases) in VAS scores[7-9], including a substantial increase at 1 year [50.2 to 71.5 (n = 31)][22] and at 10 years after LF [52.7 to 65.3 (n = 79)][7-9]. Only one study

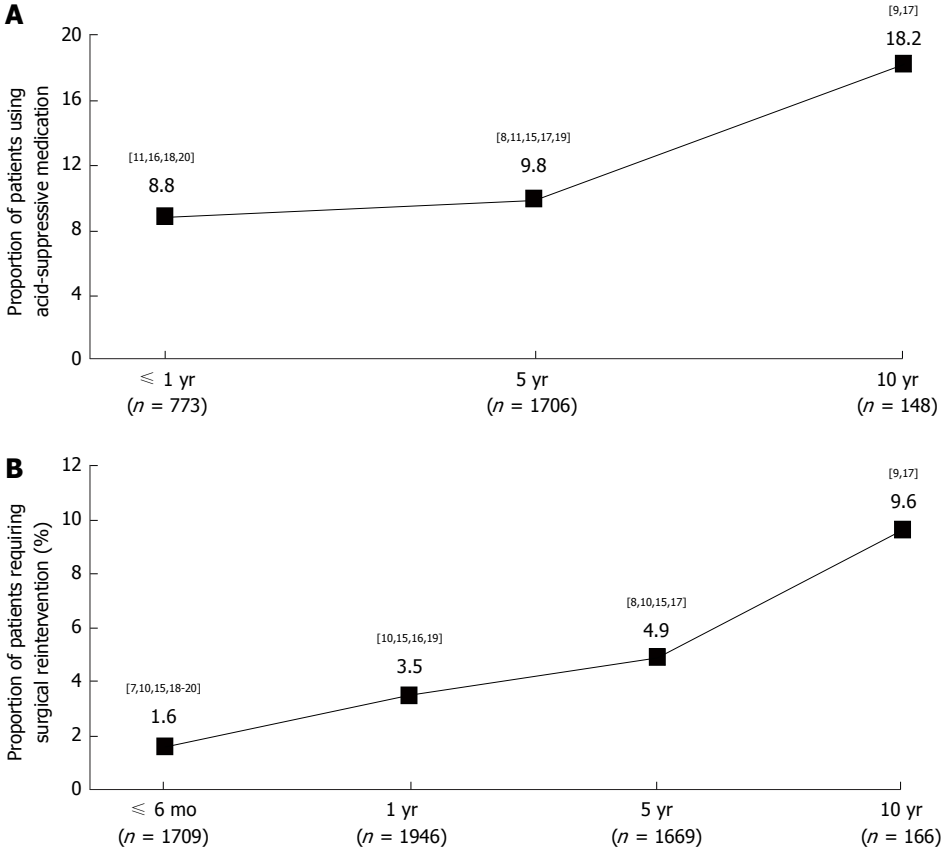

Figure 4 Proportion of patients using acid-suppressive medication (A) and requiring surgical reintervention (B) after laparoscopic fundoplication.

used the GIQLI scores and reported improved (increased) values at 1 year after LF [104 to 119 (n = 351)][16].

Other indicators of the effectiveness of laparoscopic fundoplication in partial responders

Patient satisfaction: The number of patients satisfied with the outcome of LF was reported by six trials[7-10,15,18-20]. In the year after LF, 88.8% (71/80) of patients expressed satisfaction with the procedure across two trials[18,20]; at follow-ups ranging from 3 to 5 years, 93.2% (1489/1598) of patients were satisfied across four trials[7,8,15,19]. In the only trial reporting patient satisfaction 10 years after LF, 78.5% (62/79) of patients were satisfied with the outcome of the operation[9].

Postoperative acid-suppressive medication use: Use of acid-suppressive medication after LF was reported by eight trials for up to 10 years (Figure 4A)[7-9,11,15-20]. Across four trials reporting data for the year immediately after LF, 8.8% (68/773) of patients were using acid-suppressive medication[11,16,18,20]. Across five trials reporting 5-year outcomes of LF, 9.8% (167/1706) of patients were using acid-suppressive medication[7-9,11,15,17,19]. In two trials reporting results at 10 years, 18.2% (27/148) of patients were using acid-suppressive medication[7-9,17].

Intraoperative and postoperative complications: The proportion of patients scheduled for LF surgery who during the actual operation had to be converted to open fundoplication was 7.0% (115/1652)[10,15,17,18,20]. The number of patients who had undergone LF and required subsequent surgical reintervention (to repeat LF, to perform repetitive dilatation, or to repair an intrathoracic herniation), at some point during the follow-up period, was reported by eight trials (Figure 4B)[7-10,15-20]. Across the two studies with the longest follow-up, 9.6% (16/166) of patients required surgical reintervention during the decade following the initial LF[9,17].

Impact of laparoscopic fundoplication on reflux measures in partial responders compared with complete responders

Only one trial compared partial and complete responders (classified by patient-reported symptom relief on a VAS before and after LF[11]. Symptom scores were assessed by patients rating five GERD symptoms, with each symptom scored as a product of severity and frequency. Despite similar baseline symptom scores during PPI abstinence [complete responders: 33.7 (n = 274); partial responders: 34.3 (n = 445)], the partial responders experienced significantly less symptom relief during PPI treatment before LF than complete responders (13.2 and 22.6, respectively), and also 6 mo, 2 years and 5 years after LF (values not shown). This study also reported a higher rate of postoperative PPI use in partial responders than in complete responders (16.0% and 11.0%,

respectively, at 5 years), although this difference was not statistically significant.

DISCUSSION

Across the selected studies, substantial reductions in the prevalence of heartburn and regurgitation in partial responders were observed immediately after LF compared with PPI therapy before LF. However, symptoms recurred in around 30%-35% of patients a decade after LF in those studies reporting long-term follow-up data. This coincided with an increase in the prevalence of acid-suppressive medication use from 9% to 18% over the same period. The impact of LF on the prevalence of dysphagia after LF is less clear. As expected, dysphagia may remain an issue for many patients during the first 6 mo after LF[25]. However, the prevalence of dysphagia also appeared to decrease 1 year and 5 years after LF but increased again after 10 years, suggesting a biphasic response. It seems possible that this late recrudescence of dysphagia coincides with the recurrence of heartburn and regurgitation symptoms observed over time after the operation.

Large reductions in esophageal acid exposure in partial responders were also seen after LF compared with PPI therapy alone before LF. A corresponding superiority of surgical repair in the reductions in esophageal acid exposure compared with PPI therapy alone has also been reported in complete responders 5 years after LF, in a recent study comparing long-term use of esomeprazole with surgery for treatment of chronic GERD (the LOTUS trial)[26]. The presented review suggests that surgery can elicit a further normalization of acid exposure in PPI partial responders, with a corresponding clinical improvement. These data would therefore suggest that in these patients it may be important to further minimize acid exposure in order to attain symptom control.

Additionally, increases in resting LESP of 60%-160% were observed 6 mo after LF. Furthermore, the durability of the hiatal repair appears robust in the years after LF. Some post-fundoplication problems may be a consequence of over-tightening of the sphincter by LF, but the optimal level of sphincter pressure following this procedure remains unclear. LF was found by several studies to also improve QoL according to various instruments.

Only one trial was identified that compared the effects of LF in partial responders with those in complete responders. This study clearly showed a greater benefit for LF in terms of reflux symptom scores at 5 years in the latter group[11]. This study also reported a higher rate of postoperative PPI use in partial responders than in complete responders. This albeit limited evidence lends support to the assertion that partial responders do not respond as favourably to LF as complete responders; therefore, appropriate patient selection for surgery is crucial.

Strengths and limitations

Most studies reporting the efficacy of LF include only patients who respond well to PPI treatment[3]. However, patients for whom PPI treatment does not provide adequate symptom relief are also commonly referred for surgical therapy[27]. To our knowledge, this is the first systematic review to assess the short- and long-term effects of LF in patients with GERD whose symptoms respond poorly to PPI therapy, offering the most comprehensive assessment of this important clinical question to date.

Our systematic searches did not identify any "ideal" studies (*i.e.*, RCTs comparing the results of LF in partial responders and complete responders with those of optimized PPI maintenance therapy). As such, we were forced to reduce the stringency of our study selection criteria. Another limitation of this review is the large variation in the design of the selected studies. For example, the definition of a partial response to PPI treatment in patients with GERD was often unclear and inconsistent across studies, although this is not surprising given that no consensus definition exists[6]. There was also substantial variation in the symptom definitions used and in the use of objective measures of reflux. Another weakness of the current review is that few of the studies reporting long-term follow-up data also presented baseline data. Data were therefore collated at each time point and this approach may mean that differences in trends among studies are missed. We cannot exclude the possibility that some degree of reporting bias contributes to the high prevalence of symptom recurrence after LF; patients with adequate symptom relief over time may be less likely to report for follow-up, meaning that recurrent symptoms may be relatively over-reported. Regardless, it is clear that a substantial proportion of partial responders exhibit GERD symptoms 10 years after LF. Despite the limitations of the included studies, useful inferences can be drawn about the effectiveness of LF in partial responders, and about recommendations for future studies in this area.

Clinical implications

The data presented provide strong evidence that LF is superior to acid-suppressive medication at reducing reflux symptoms in partial responders to PPI therapy, but that these symptoms may recur and acid-suppressive medication use may increase in a substantial proportion of patients over time. Furthermore, although data are limited, LF appears to be less effective in patients with GERD whose symptoms only partially respond to PPI therapy than in complete responders to PPI therapy. Indeed, these results support the findings of a recent study that concluded that the response of symptoms to preoperative PPI treatment is an excellent predictor of the response of symptoms to fundoplication[28]. This may have implications for recent recommendations for LF as an alternative to the high costs of continuous acid-suppressive medication[29], because they are based mainly on data from complete responders and may thus overestimate the effectiveness of LF in partial responders. These factors must be carefully considered, especially in light of recent

improvements in drug pharmacokinetics that may shift the balance towards pharmacological therapy, depending on the age and health status of the patients, and the pathology of GERD.

In a limited time horizon, LF offers a substantial and clinically relevant improvement in GERD symptoms, physiological measures of GERD and QoL parameters in partial responders beyond that provided by PPI treatment alone. However, in the long term a substantial proportion of these patients experience a recurrence of GERD symptoms. Limited data also suggest that LF is less effective at reducing symptoms in partial responders than in complete responders. This may affect cost arguments for using LF rather than acid-suppressive medications because data are based largely on complete responders.

COMMENTS

Background

When patients with gastroesophageal reflux disease (GERD) do not respond adequately to proton pump inhibitor (PPI) treatment, they are often referred for surgery (laparoscopic fundoplication). However, justification for this surgery is largely based on trials evaluating patients who respond well to PPIs.

Research frontiers

This review aimed to systematically assess the available evidence for this procedure in patients responding only partially to PPI therapy (partial responders).

Innovations and breakthroughs

This is the first review to assess the available evidence for efficacy of laparoscopic fundoplication in this subset of patients who do not respond adequately to PPI treatment.

Applications

It is hoped referral for laparoscopic fundoplication can be made on an objective patient-by-patient basis, with pre-surgery response to PPI treatment taken into consideration. This review also outlines inconsistencies in patient definitions, and suggests preferred outcomes and methods of reporting.

Terminology

Histamine-2 receptor antagonists: acid-suppressive medication often used to treat GERD. PPI: acid-suppressive medication often used to treat GERD. Laparoscopic fundoplication: a minimally invasive surgical procedure often used to treat GERD; the surgeon accesses *via* a small incision in the abdomen, and partially or completely wraps the patient's fundus (the upper part of the stomach) around the lower esophageal sphincter, to minimize the degree of reflux.

Peer review

The paper identifies that GERD symptoms improve after laparoscopic fundoplication, but subsequently recur, and acid-suppressive medication use increases. The research methodology is well written and well organized.

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BRIEF ARTICLE

Epidemiological and clinical characteristics of inflammatory bowel diseases in Cairo, Egypt

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Abstract

AIM: To study the natural history, patterns and clinical characteristics of inflammatory bowel diseases (IBD) in Egypt.

METHODS: We designed a case-series study in the gastroenterology centre of the Internal Medicine department of Cairo University, which is a tertiary care referral centre in Egypt. We included all patients in whom the diagnosis of ulcerative colitis (UC) or Crohn's disease (CD) was confirmed by clinical, laboratory, endoscopic, histological and/or radiological criteria over the 15 year period from 1995 to 2009, and we studied their sociodemographic and clinical characteristics. Endoscopic examinations were performed by 2 senior experts. This hospital centre serves patients from Cairo, as well as patients referred from all other parts of Egypt. Our centre received 24156 patients over the described time period for gastro-intestinal consultations and/or interventions.

RESULTS: A total of 157 patients with established IBD were included in this study. Of these, 135 patients were diagnosed with UC (86% of the total), and 22 patients, with CD (14% of the total). The mean ages at diagnosis were 27.3 and 29.7, respectively. Strikingly, we noticed a marked increase in the frequency of both UC and CD diagnoses during the most recent 10 years of the 15 year period studied. Regarding the gender distribution, the male:female ratio was 1:1.15 for UC and 2.6:1 for CD. The mean duration of follow up for patients with UC was 6.2 ± 5.18 years, while the mean duration of follow up for patients with CD was 5.52 ± 2.83 years. For patients with UC we found no correlation between the severity of the disease and the presence of extraintestinal manifestations. Eleven patients had surgical interventions during the studied years: 4 cases of total colectomy and 7 cases of anal surgery.

CONCLUSION: We observed a ratio of 6:1 for UC to CD in our series. The incidence of IBD seems to be rising in Egypt.

Key words: Natural history of Inflammatory bowel diseases; Epidemiology of Ulcerative colitis; Epidemiology of Crohn's disease; Epidemiology of inflammatory bowel diseases in Egypt; inflammatory bowel diseases Prevalence; Incidence of ulcerative colitis; Incidence of Crohn's disease

Core tip: The precise aetiology of inflammatory bowel disease (IBD) remains obscure. In our study, the ratio of patients diagnosed with ulcerative colitis (UC) to those diagnosed with Crohn's disease (CD) was approximately 6:1. The total colectomy rate in our study was 2.9%, after a follow up period of 5-15 years, which is far lower than the rates of Western countries. We found that the characteristics of IBD in the Egyptian popula-

tion were more similar to Asian and African IBD patterns. We noticed a marked increase in the frequency of UC and CD diagnoses over the past 10 years, which indicates an increasing incidence of IBD in Egypt.

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INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) are collectively referred to as inflammatory bowel diseases (IBD)[1]. They mainly affect young populations, majorly altering their quality of life and increasing morbidity, compared to the general population. Although the exact aetiology of IBD has still not been exactly identified[2-5], it is believed that the pathogenesis of IBD includes immune deregulation secondary to environmental factors in genetically susceptible individuals. This results in a mounted immune response to the normally existing intestinal flora or epithelial antigens[2,6-8].

IBD occur with different frequencies around the world. The countries reporting for the highest incidence of UC are the United States, the United Kingdom and Sweden[9-11].

IBD have always seemed to be rare in the Middle East and Northern Africa. No accurate registry or cohort of patients had ever studied the exact prevalence of CD and UC in these populations. In Mediterranean countries, the prevalence of UC was estimated at 5/100000 in urban areas[12].

Recent data from a few single-centre studies have pointed to a change in the disease incidence that is usually explained by lifestyle changes, such as urbanisation, and changes in alimentary habits, such as greater consumption of fast food, greater consumption of carbohydrates, and a lower daily intake of alimentary fibres.

In a recent review of the natural history of IBD, it was noted that as countries become Westernised, the incidence of UC increases first and is later followed by CD[13-15]. Asia had a high ratio of UC/CD incidence in the 1980s and 1990s, but in 2000, the incidence of CD increased. Both diseases have emerged in countries in which they had rarely been previously reported, including Japan, South Korea, India, Iran, Lebanon, Thailand, the French West Indies, and North Africa[16-18]. In these countries, the occurrence of UC preceded that of CD by approximately 10 years. The overall incidence of IBD can be broken down into several geographic zones: those with a high incidence, those with a moderate incidence, those with low incidence 15 years ago but with a consistently increasing incidence, and those with an unknown incidence[13].

Overall, a pattern can be drawn for IBD frequency in the developing world: first, a low UC incidence; then, an increase in UC, while the CD incidence remains low; and finally, a CD incidence that approaches UC levels.

In this study, we studied the sociodemographic and clinical characteristics of patients diagnosed with CD and UC in the gastroenterology centre of the Internal Medicine department of Cairo University, which is a tertiary care referral centre in Cairo. To the best of our knowledge, this is the first trial establishing a cohort of IBD patients and starting a registry for data collection and analysis in Egypt.

MATERIALS AND METHODS

The study aimed to identify the socio-demographic and clinical characteristics of IBD patients in a very diverse population (Cairo agglomeration).

In the Middle East, as well as in most of the African countries, data on IBD patients are lacking, and there are no solid databases or registries to follow up the pattern of the disease.

We included all patients in whom the diagnosis of UC or CD was confirmed by clinical, laboratory, endoscopic and histological examination over the 15 year period from 1995 to 2009.

Our hospital gastroenterology centre serves patients from Cairo and also patients referred from all other parts of Egypt. Our centre received 24156 patients over the same duration of time who were referred for gastrointestinal consultations and/or interventions.

First, we identified the presenting complaint of the patients who consulted our centre and the reason for which the endoscopic exploration was ordered. We considered patients presenting with chronic diarrhoea, rectal bleeding, recurrent abdominal pains or discomfort, melena, weight loss, and/or perianal fistula or abscess. The diagnosis of IBD was established by clinical, endoscopic, histological, and/or radiological criteria.

CD was diagnosed if skip lesions were found at endoscopy; a cobblestone appearance was evident; mucosal ulceration was found upon colonoscopy; or aphthous lesions were found at endoscopy. Deep inflammation or chronic terminal ileal inflammation, with or without radiologic evidence of skip lesions, stricturing disease, fistulising disease, existence of perianal disease (skin tags, abscess, fistula), small intestinal involvement or non-caseating granulomas, was also included in the diagnosis. Endoscopic examinations were performed by 2 senior experts.

Extraintestinal manifestations included musculoskeletal, mucocutaneous, hepatic, ophthalmic, and urinary tract involvements.

UC was diagnosed when there was evidence of a diffuse mucosal disease of colon with different proximal extensions from the rectum, superficial inflammation, crypt abscess, cryptitis, and rectal involvement without any evidence of small bowel involvement other than backwash ileitis.

We included patients who had an established diag-

Table 1 Demographic characteristics of ulcerative colitis patients in Cairo

	Ulcerative colitis patients
Mean age at presentation (yr)	27.3 (± 12)
Mean age at presentation (yr) (male:female)	29.2 (± 13.4):25.7 (± 10.6) $P = 0.093$
Mean age at presentation (yr) (urban:rural)	25.9 (± 9.7):27.9 (± 12.7) $P = 0.394$

nosis of IBD over the 15 years from 1995 to 2009 and who were referred to our centre. The following data were gathered for assessment: demographics, clinical features, area of residency, living conditions (city or countryside), smoking, family history of IBD, disease characteristics, extraintestinal manifestations, medical treatment used, and surgical interventions. Clinical information was obtained from medical records and patient interviews.

A diagnosis of IBD was established according to the corresponding criteria. For cases of UC, the true love classification was used to assess severity, and the Montreal classification was used to assess the extent of the disease[19]. Endoscopic grades were assigned in the form of mild (erythematous oedematous rectal mucosa, absent or distorted vascular pattern), moderate (marked oedema, spontaneously bleeding mucosa, purulent exudates) and severe (frank ulcerations) degrees. The histopathological findings included the following: vascular congestion, crypt abscesses, mucin depletion, cellular infiltrate, cryptitis, and crypt branching.

For the CD cases, the Crohn's disease activity index was used to assess the disease activity. The Montreal classifications and endoscopic grades assessed the activity as follows: (1) inactive (the vascular pattern is only slightly distorted and there is, fine granularity without friability or epithelial defects); (2) mildly active (there is unequivocal erythema, either focal or confluent, and some friability without epithelial necrosis); (3) moderately active (a few aphthoid erosions or small ulcers are noted); or (4) severe (ulcers are larger and more numerous). The histopathological findings included the following: cellular infiltrate, focal inflammation, microfistulisation, non-caseating granulomas, cobblestoning, and lymphoid hyperplasia.

Finally, as Egypt is currently in an endemic for parasitic infestations, a stool analysis was performed for all patients; we only included patients with non-complicated or evolving parasitic infections.

Statistical analysis

Data analysis was performed by the χ^2 test, and statistical significance was set at a P value of 0.05. The protocol of this study was approved by the review board of the department of Internal Medicine, according to the Declaration of Helsinki.

RESULTS

A total of 157 patients with established IBD were included in this study. From those, 135 patients were diagnosed with UC (86% of the total), and 22 patients, with CD (14% of the total). The mean ages at diagnosis were, respectively, 27.3 and 29.7. The mean age of diagnosis for UC patients residing in Cairo was 27.9, while it was 25.9 years for those living outside of Cairo, with no statistically significant difference (Table 1).

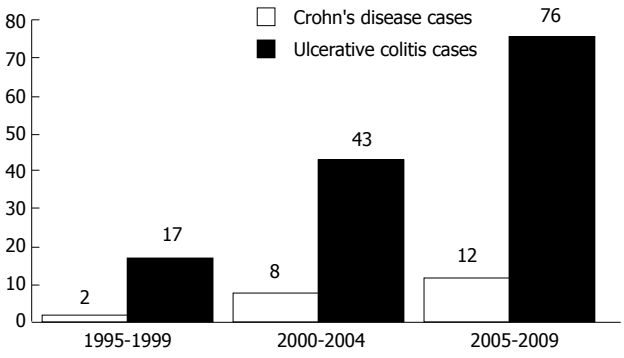

Figure 1 Number of ulcerative colitis and Crohn's disease cases over the first, second and last five years of the 15 years duration.

As for gender distribution, the male: female ratio was 1:1.15 for UC and 2.6:1 for CD. Among UC patients, only 38 patients (27.9% of the total) were not residents of Cairo (rural area of residency). As for CD, they were almost all residents of Cairo (except for one patient). Being a resident of Cairo or not was not correlated with the type of disease (UC or CD).

Strikingly, we noticed a marked increase in the frequency of both CD and UC diagnoses in the last 5 years, stronger than had occurred in the middle five years and much stronger than in the first 5 years, as shown in Figure 1. We analysed the association of cigarette smoking with both diseases; for UC patients, there were 121 patients with no active smoking habits at the date of diagnosis (88.9% of the total). For CD, only 2 patients were active smokers at the date of diagnosis.

For patients with UC, we found no correlation between the severity of the disease, the presence of extraintestinal manifestations (especially hepatobiliary associations) in either the area of residency or the smoking habits.

The mean duration of follow up for patients with UC was 6.2 ± 5.18 years, while for CD patients, the mean duration of follow up was 5.52 ± 2.83 years. There was no statistically significant difference between either the genders or between those patients residing in or outside of Cairo. For UC patients residing in Cairo, the mean duration of follow up was 6.2 years, while it was only 6 years for those living outside of Cairo, with no statistically significant difference (Tables 2 and 3).

We studied all of the endoscopic findings (Table 4), and we did not find any statistically significant correlations among the area of residency, the gender, the age of presentation of the UC and active smoking with either the severity of the lesions or with the extent of the disease.

Table 2 Sociodemographic and clinical data of ulcerative colitis patients

Total number	135
Gender (M/F)	63/72
Age at first presentation (mean ± SD)	27.3 ± 11.7
Resident of Cairo (yes/no)	97/38
Smoking (yes/no)	14/121
Main presenting symptoms included: rectal bleeding, diarrhea, mucous, abdominal pain, loss of weight, fever	128/113/72/76/8/8
Follow up duration (mean ± SD)	6.2 ± 5.18
Severity of symptoms (true love classification) mild/moderate/severe	66/52/17
Anoperineal lesions (yes/no)	6/129
Hepatobiliary or pancreatic manifestations No/Fatty liver/sclerosing cholangitis	127/7/1
Other extra intestinal manifestations No/conjunctivitis/arthralgia/both	109/10/16/25
History of appendicectomy (yes/no)	18/117
Family history of UC (yes/no)	2/135
Extent of colitis Proctitis/left side colitis/pancolitis	25/88/22
Types of intestinal infections detected (total 31) Entameba histolytica/giardiasis/schistosomiasis	23/5/3

M: Male; F: Female; UC: Ulcerative colitis.

Table 3 Sociodemographic and clinical data of Crohn's disease patient

Total number	22
Gender (M/F)	16/6
Age at first presentation (mean ± SD)	29.72 ± 12.13
Resident of cairo (yes/no)	21/1
Smoking (yes/no)	2/20
Main presenting symptoms included: diarrhea, colics, fever, WT loss, rectal bleeding, vomiting	22/18/9/10/6/4
Follow up duration (mean ± SD)	5.52 ± 2.83
Severity of symptoms (mild/moderate/severe)	9/11/2
Anoperineal lesions (yes/no)	8/14
Extra intestinal manifestations (yes/no)	8/14
No/fatty liver/arthralgia/arthralgia and uveitis	14/1/7/3
History of appendicectomy (yes/no)	3/19
Montreal classification:	
A1/A2/A3	2/14/6
B1/B2/B3 /P>>	16/5/1/8
L1/L2/L3/L4>>	6/3/13/2
Endoscopic grading (mild/moderate/severe)	4/13/5
Follow up endoscopy (stationary/improved/ progressed/did not do)	2/15/2/3
Localization of sites of CD affection Ileal/ileocolonic/colonic/oesophageal	6/13/3/2
Types of intestinal infections detected (total 9) Entameba histolytica/schistosomiasis	7/2

M: Male; F: Female; CD: Crohn's disease.

The pathological interpretation of the examined biopsies taken during endoscopic examinations for all the patients concluded with changes typical of the disease (Tables 5 and 6).

In our series, only 11 patients had surgical interventions during the years of follow up, which were either a total colectomy (4 UC patients) or an anal surgery (7 CD patients). The type of medical treatment, number of relapses and laboratory findings at the time of diagnosis are shown in Tables 5 and 6.

Only two patients had family history of IBD, and only 18 patients had previous history of appendicectomy prior to the presentation of UC. Neither of these was correlated with the age at presentation, the gender of the patient or the area of residency.

We did not confirm any malignant changes in the series of patients followed in our centre, except for one patient who developed colorectal carcinoma on follow-up. In our series, 40 patients had a non-evolving and non-complicated parasitic infection.

DISCUSSION

The exact aetiology of IBD remains obscure; many epidemiologic studies in different populations have shown an environmental and a genetic role in CD and UC. These studies have reported that the rate of this disease is higher in Scandinavian countries, Great Britain, Canada, and the United States than in Central Europe, Africa, or Asia. Our series included a small number of patients, which could have reflected either a weak incidence or prevalence of the disease in this population or a misdiagnosis of patients presenting with symptoms suggestive of IBD (trying to find another explanation for such a presentation).

The mean age of diagnosis for UC patients residing in Cairo was 27.9, while the mean age for those living outside Cairo was 25.9, with no significant difference. However, these findings cannot allow us to conclude that differences exist in the incidence of the disease between the urban and rural populations of Egypt.

The male: female ratio was 1:1.15 for UC, which is similar to most other studies[20-22] (with a slight increase of the female prevalence, denoting an increased number of affected females compared to other parts of the world). However, for CD, the male: female ratio was 2.6:1, denoting a male predominance similar to the results of a study from Tunisia, which showed a male predominance in CD[23], and similar to the results of epidemiological studies from Japan and China[24]. In contrast, studies from North America, Sweden and Northern France showed a female predominance in CD[24], and a recent study from Kuwait concluded that CD is equally common in males and females[25]. Several reasons may explain these differences, including the possibility that the gender ratios in CD are highly dependent on age, as well as geographic region[24].

In our study, the ratio of patients diagnosed with UC to patients diagnosed with CD was approximately 6:1. This was similar to results observed from different parts of the world, where UC is much more common than CD. In northeastern Poland, this ratio was approximately 15:1, and an increase in the total number of cases diagnosed with IBD has been reported[26]. Additionally, reports from Greece, Hungary, China and Lebanon confirm that CD is diagnosed less frequently[27].

Lifestyles may contribute to the expression of UC.

Table 4 Clinical scoring of the endoscopic findings of ulcerative colitis patients according to the Montreal classification

Number of patients/extent of colitis (endoscopic grading according to the Montreal classification)	E1 (Proctitis) (25 patient, 18.5%)	E2 Left side colitis (88 patient, 65.2%)	E3 Pancolitis (22 patient, 16.3%)
Number of patients/severity of colitis (endoscopic grading according to the Montreal classification)	Mild (25 patient, 18.5%)	Moderate (94 patient, 69.6%)	Severe (16 patient, 11.9%)
Follow up endoscopic grading Stationary/improved/progressed/did not do			20/83/6/26

Table 5 Type of treatment, relapses, histopathological and laboratory findings of ulcerative colitis patients

Treatment used: (single drug or combinations) Oral 5-ASA ±, 5-ASAenemas or supp., prednisone, azathioprine, corticosteroids enemas. (Infliximab was used in 2 cases)	
Surgical interference (yes/no)	4/131
Relapses (mean ± SD)	1.514 ± 1.575
Relapses (median/minimum/maximum)	1/0/10
Malignant transformation	1
Mean ESR 1st hour (mean ± SD)	36.37 ± 24.73
CRP (positive/negative/not done)	39/34/60
ANCA (positive/negative/not done)	12/28/95
ASCA (positive/negative/not done)	8/31/96
Pathological findings included: Vascular congestion, Crypt abscesses, Mucin depletion, Cellular infiltrate, Cryptitis, Crypt branching	
Pathology (diagnostic/suggestive/non conclusive)	78/43/14
Mean Hemoglobin concentration, gm/dL (mean ± SD)	11.2 ± 2.8
Mean PLT count (mean ± SD)	335428.6 ± 140119.1
Mean TLC (mean ± SD)	8875 ± 4059.67

ASA: Aminosalicylic acid; CRP: C-reactive protein; ANCA: Anti-neutrophil cytoplasmic antibodies; ASCA: Anti-Saccharomyces cerevisiae antibodies.

Table 6 Type of treatment, relapses, histopathological and laboratory findings of Crohn's disease patients

CDAI (mean ± SD)	108.21 ± 53.84
CDAI (median/minimum/maximum)	103.33/38/259.5
Treatment used: (single drug or combinations) oral 5-ASA ± Metronidazole, ciprofloxacin, azathioprine, prednisone, infliximab, surgical interference (yes/no)	7/15
Relapses (mean ± SD)	1.68 ± 2.21
Relapses (median/minimum/maximum)	1/0/8
Malignant transformation	None
Mean ESR 1st hour (mean ± SD)	49.52 ± 30.98
CRP (positive/negative)	17/5
ANCA (positive/negative/not done)	6/16
ASCA (positive/negative)	4/18
Pathological findings included: cellular infiltrate, focal inflammation, microfisttulization, non caseating granulomas, cobblestoning, lymphoid hyperplasia	
Pathology (diagnostic/suggestive/non conclusive)	7/8/7
Mean hemoglobin concentration, gm/dL (mean ± SD)	11.95 ± 2.14
Mean PLT count (mean ± SD)	296272.72 ± 146308.60
Mean TLC (mean ± SD)	7868.57 ± 2708.17

CDAI: Crohn's disease activity index; ASA: Aminosalicylic acid; CRP: C-reactive protein; ANCA: Anti-neutrophil cytoplasmic antibodies; ASCA: Anti-Saccharomyces cerevisiae antibodies.

Such factors as smoking, drinking tea and adhering to vegetarian diets have a protective effect against UC, while ex-smoking, psychological stress and family history of UC are shown to be risks for an increased incidence of IBD[20,28-31].

In our study, we did not find any correlation between smoking habits and the occurrence or severity of UC because most of our patients in this study did not have a history of smoking at the time of diagnosis. Additionally, there was no correlation between smoking and the occurrence of extra-intestinal manifestations.

It is generally thought that a family history of UC increases an individual's risk of developing UC. We failed to find this in our study, as a family history was reported in only two patients.

This may be explained by the under diagnosis of these diseases due to a low disease awareness; to the confusion of IBD with the causes of infectious diarrhoea, which is common in our country; and to limited access to diagnostic tools as a result of the limited resources available in community health centres.

The clinical characteristics of the disease in our study showed that most of our patients with UC had mild distal or left-sided colitis (approximately 85%), confirmed clinically and endoscopically, and that most responded well to medical treatment. Only a few patients were referred for surgical intervention. Throughout the follow up period for UC, 83 patients (61.5%) showed an improvement in the disease activity; 20 patients retained the same activity score (14.8%); the disease activity progressed in 6 patients (4.4%); and 26 (19.2%) patients did not submit to a follow up endoscopy but also did not show any clinical relapse. The total colectomy rate in our study was 2.9% after 5-15 years, which is far lower than the total colectomy rates in studies from Western countries, which ranged from 24% to 34% after 10 years[32-37]. On the other hand, the total colectomy rate after 5-15 years in a study from South Korea was 3.3%[38]. These data indicate that the behaviour of UC in our Egyptian patients is milder than its behaviour in Western countries, more closely approximating that in the Asian population.

Although a recent study from the Middle East showed a different pattern of clinical characteristics, with more patients having pancolitis 45.5%[27], most of the data from the Asian and European regions reflected a very similar population description[26,39-41].

We also noted in our series that the rectal form of UC represented approximately 30% of cases-not far from 40%-which may indicate a good recruitment of all cases of UC, including those with the early forms of the

disease (not only the advanced or complicated cases).

Importantly, our data showed that a marked increase in the diagnoses of both UC and CD occurred in the past 5 years, resulting in an incidence greater than the previous 5 years and much greater than that of the 5 years before that. These data are shown in Figure 1, which indicates the increasing incidence of IBD in Egypt. This increasing incidence is also supported by a recent study in 2012 by Molodecky *et al*[42], who concluded that the incidence and prevalence of IBD have been increasing with time in different regions around the world, even in developing countries as they became more industrialised. The increased awareness of IBD and improvements in the necessary diagnostic tools, especially endoscopes, over the last 10 years in Egypt may be an additional factor affecting the increased frequency of IBD diagnoses.

Ruyssers *et al*[43] discussed in depth the hygiene hypothesis, which proposes a converse relationship between parasitic infections and the incidence of IBD. Epidemiological, experimental, and clinical data corroborate the knowledge that helminthes provide protection against IBD. Therefore, the use of helminth-derived molecules may result in a protective effect[43].

In our study, the parasites identified among the IBD patients (157) were Entameba histolytica (19.1%), Giardiasis (3.2%), and Schistosomiasis (3.2%), while the parasites identified in patients without IBD (23998) were Entameba histolytica (20.1%), Enterobius vermicularis (8.3%), Giardiasis (7.2%), Schistosomiasis (5.4%), Ascaris lumbricoides (2.1%), Ancylostoma duodenale (2.1%), Trichuris trichiura (1.1%), Hymenolepis nana (1%) and mixed infections (3%). These data support the hygiene hypothesis, as the exposure to helminthes in patients without IBD was much higher than in those with IBD. The findings of several recent studies provide evidence for the role of helminthes in protecting against IBD[44-51].

In conclusions, in our experience, we found that the epidemiological characteristics of IBD in Egyptian population closely resembled those of the Asian and African patterns of IBD. UC was more common than CD, and the mean age at presentation was in the late twenties. UC was more common in females, while CD was more common in males. No correlation with active smoking was found at the time of presentation. We noticed a marked increase in the frequency of IBD diagnoses IBD in the last 10 years. We observed a ratio of 6:1 for UC to CD in our series, although the global natural course of IBDs in other countries may predict a future rise in CD in Egypt and other Middle Eastern countries, such that its incidence equals that of UC. We believe that the present moment is critical in assessing the pattern of IBD spreading in Egypt, and the current status should be further studied by more exhaustive database and registry documentations of IBD patients and their characteristics.

We believe that this work should direct the further identification of factors correlating with the severity and extent of UC in African, Arab and Asian populations. Exposure to pollutants in the environment, parasitic infestations, and changes of the life styles and food habits have all been proposed to theoretically account for the observed increase in the incidence of IBD in those communities; these hypotheses require further study and focus.

COMMENTS

Background

Inflammatory bowel diseases (IBD) include ulcerative colitis (UC) and Crohn's disease (CD), both are chronic inflammations of the gastrointestinal tract. UC involve only the colon. On the other hand CD can involve any part of the gastrointestinal tract from the mouth to the anus. They mainly affect young populations with major effect on the quality of life and increased morbidity compared to general population. IBD have been considered a disease of Western countries with a pathogenesis related to the Western lifestyle. Studies showed that the incidence of IBD is now increasing in developing countries.

Research frontiers

The available data in the literature regarding IBD in North Africa are limited; therefore, the aim was to study the natural history of IBD in Egypt and to compare its clinical pattern with those in other regions including Western countries.

Innovations and breakthroughs

In the present study, the authors showed a marked increase in the frequency of diagnosis of both UC and CD in the last 10 years. These results suggests that the characteristics of IBD in Egyptian population are near to Asian and African pattern of IBD and the behavior of the disease is milder than in Western countries.

Applications

The study results suggest that the incidence of IBD seems to be rising in Egypt and may predict a future rise in IBD incidence in Egypt and other Middle East countries. This work should direct further studies with more exhaustive database and registry documentation of IBD patients and their characteristics in Egyptian, Arab and Asian population.

Terminology

True love classification: Classify the severity of UC according to the severity of symptoms to mild, moderate, and severe. Montreal classification: Classify disease phenotypes in CD based on the age at presentation, disease location and disease behavior.

Peer review

It is a very informative epidemiology study, worth of being published.

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BRIEF ARTICLE

Impairment of secondary peristalsis in Barrett's esophagus by transnasal endoscopy-based testing

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Abstract

AIM: To investigate dysfunctions in esophageal peristalsis and sensation in patients with Barrett's esophagus following acid infusion using endoscopy-based testing.

METHODS: First, physiological saline was infused into the esophagus of five healthy subjects, at a rate of 10 mL/min for 10 min, followed by infusion of HCl. Esophageal contractions were analyzed to determine whether the contractions observed by endoscopy and ultrasonography corresponded to the esophageal peristaltic waves diagnosed by manometry. Next, using nasal endoscopy, esophageal sensations and contractions were investigated in patients with, as well as controls without, Barrett's esophagus using the same infusion protocol.

RESULTS: All except one of the propulsive contractions identified endoscopically were recorded as secondary peristaltic waves by manometry. Patients with long segment Barrett's esophagus (LSBE) tended to have a shorter lag time than the control group, although the difference did not reach statistical significance (88 ± 54 s *vs* 162 ± 150 s respectively, $P = 0.14$). Furthermore, patients with LSBE had significantly fewer secondary contractions following the infusion of both saline and HCl than did either the control group or patients with short segment Barrett's esophagus (4.1 ± 1.2 *vs* 8.0 ± 2.8, $P < 0.001$ and 7.3 ± 3.2, $P < 0.01$, respectively, following saline infusion; 5.3 ± 1.2 *vs* 8.4 ± 2.4 and 8.1 ± 2.9 respectively, $P < 0.01$ for both, following infusion of HCl).

CONCLUSION: Using nasal endoscopy and a simple acid-perfusion study, we were able to demonstrate disorders in secondary peristalsis in patients with LSBE.

Key words: Barrett's esophagus; Transnasal endoscopy; Acid infusion test; Esophageal sensation; Secondary peristalsis

Core tip: We have developed a simple technique for esophageal examination based on transnasal endoscopy in unsedated patients. First, manometric waves and esophageal contractions were evaluated using three different modalities following the infusion of acid into the lower esophagus. Next, using nasal endoscopy, esophageal contractions and sensations were investigated in patients with Barrett's esophagus. It was possible to observe secondary peristalsis endoscopically, using nasal endoscopy and a simple acid-perfusion study, we were able to demonstrate disorders in secondary peristalsis in patients with long segment Barrett's esophagus.

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INTRODUCTION

Because of its increasing prevalence in Asian countries[1,2], the clinical impact of gastroesophageal reflux disease (GERD) is also increasing. Furthermore, some of the patients with GERD will go on to develop Barrett's esophagus, which itself can progress to adenocarcinoma[3]. Both GERD and Barrett's esophagus result from chronic injury following long-term exposure of the squamous mucosa to gastric acid or bile. Pathogenic exposure to refluxate may be due to disturbances in anti-reflux barriers or delayed luminal acid clearance because of abnormalities in esophageal motility or the sensory system. However, the precise causative dysfunction varies between patients; thus, esophageal function tests are needed to determine the cause to enable cause-specific treatment. However, such examinations, including manometry[4-7], sensory testing[8-10], and pH monitoring[11-14], are not routinely used in clinical practice because they are tedious, complicated, and invasive.

We have developed a simple and versatile technique for esophageal examination based on transnasal endoscopy in unsedated patients. The test can simultaneously evaluate either structural abnormalities of the lumen and anti-reflux barriers or dysfunctions in esophageal peristalsis and sensation induced by acid infusion. Using this endoscopy-based test in the present study, we examined patients with Barrett's esophagus and healthy controls to identify abnormalities in esophageal function related to Barrett's esophagus.

MATERIALS AND METHODS

Study design and subjects

The present study was approved by the Ethics Committee of The Jikei University School of Medicine (Tokyo, Japan) and was conducted at Jikei University Hospital.

Preliminary study evaluating manometric and endoscopic assessments of peristalsis and propulsive contractions, respectively

Five healthy subjects without obvious GERD symptoms [all men; age (mean ± SD) 29 ± 3 years] were recruited for the preliminary study. All subjects provided written informed consent before participating in the study. Simultaneous manometry and endoscopy-based testing were performed in these subjects to evaluate physiological saline and acid infusion-induced contractions of the esophagus (Figure 1). An ultrathin endoscope (XP 260 N; Olympus Medical Systems, Tokyo, Japan) was inserted transnasally, without sedation, with the tip of the scope located in the lower esophagus, approximately 5 cm oral from the esophagogastric junction (EGJ). Endoscopic

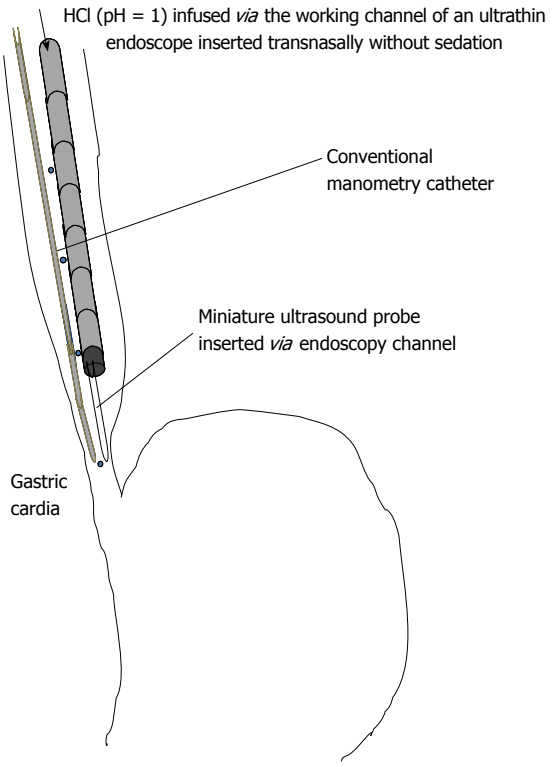

Figure 1 Esophageal contractions induced by physiological saline and acid infusion were analyzed simultaneously using three different modalities, namely conventional manometry, endoscopic ultrasonography and endoscopic observation.

ultrasonography was performed using a radial-type miniature probe (Model UM-S20-17S, 20 MHz; Olympus Medical Systems). The probe was inserted *via* the endoscope channel and positioned in the lower esophagus approximately 2 cm oral from the EGJ. Conventional manometry was performed using a POLYGRAF ID (Alpinebiomed, Los Angeles, CA, United States) and an infusion pressure four-channel catheter (4.5 mm outside diameter), with an aperture and pressure converter in the 5-cm space (Zinetics, Salt Lake City, UT, United States). To evaluate contractions elicited by the infusion of physiological saline and acid, indigo carmine was used to color both the physiological saline and acid solution. The colored physiological saline was infused initially into the esophagus at a rate of 10 mL/min for 10 min *via* the working channel of the scope using an autoinfusion pump (TE-171; TERUMO, Tokyo, Japan). Subsequently, and without alerting the healthy subject to the change, the colored HCl (pH = 1) was infused for another 10 min at the same rate[15]. he manometric waves and ultrasonographic and endoscopic views were displayed on the same monitor using a screen separation device (MV-410RGB; HOUEI, Tokyo, Japan).

Esophageal contractions induced by the infusion of physiological saline and acid were analyzed simultaneously by all three modalities, namely conventional manometry, endoscopic ultrasonography, and endoscopic observation. This was done to determine whether the esophageal peristaltic waves diagnosed by manometry

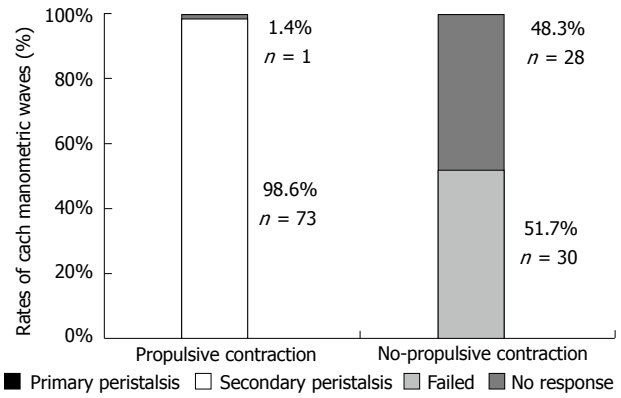

Figure 2 Comparisons of results of endoscopic evaluations [propulsive (*n* = 74) and non-propulsive (*n* = 58) contractions] and manometry (primary peristalsis, secondary peristalsis, failed, or no response).

corresponded to the propulsive contractions identified by endoscopy. In the present study, primary and secondary peristalsis were defined as successful if a pressure wave > 12 mmHg at the two proximal esophageal recording sites [12 and 17 cm above the lower esophageal sphincter (LES)] and > 25 mmHg at the middle and distal esophageal recording sites progressively traversed all the esophageal recording sites[16]. Peristaltic progression was defined as a peristaltic velocity of ≤ 6 cm/s[17]. Failed peristalsis was defined as either failure to generate a pressure wave > 12 mmHg at the two proximal esophageal recording sites and > 25 mmHg at the middle and distal esophageal recording sites, a failure of the wave to traverse each of the esophageal recording sites, or a peristaltic velocity > 6 cm/s between the recording sites 2 and 17 cm above the LES[16]. Esophageal contractions were determined to be propulsive if a full contraction observed by endoscopic sonography propelled the indigo carmine-stained acid retained in the esophagus into the stomach. Esophageal contractions were determined to be non-propulsive if an incomplete contraction did not propel the acid into the stomach. Peristaltic waves and propulsive contractions were identified as primary or secondary on the basis of the presence or absence of deglutition, respectively.

Primary study evaluating acid-induced peristalsis and sensation in the esophagus

After we confirmed the concordance of esophageal peristalsis as assessed by manometry and propulsive contraction determined endoscopically, we performed the primary study using endoscopy-based testing without manometry. The ultrathin endoscope was inserted transnasally in unsedated patients to allow for endoscopic observation of the esophagus and cardia. Then, esophageal propulsive contractions and esophageal sensations were assessed simultaneously during infusion of physiological saline and HCl (pH = 1) (10 min each; 10 mL/min) *via* the endoscope channel. A 5-min interval was allowed between the acid and saline infusions.

The endpoints used to assess esophageal sensations following infusion were lag time, intensity rate, and the acid perfusion sensitivity score (APSS). Lag time was defined as the time (in seconds) to the initial perception of typical symptoms, such as heartburn. The intensity of symptoms associated with acid perfusion was evaluated using a previously validated verbal descriptor scale, with symptoms scored on a scale of 0-10, where 0 means no symptoms and 10 means strong symptoms.The APSS was calculated from the duration of typical symptom perception, expressed in seconds, and the sensory intensity rating at the end of the acid perfusion. The APSS was divided by 100 for convenience[15].

This primary study was conducted in patients with Barrett's esophagus and subjects undergoing a planned endoscopic examination as part of a routine health check. All subjects provided written informed consent. Using endoscopy-based testing, we evaluated propulsive contractions and acid-induced sensations in these subjects to identify any abnormalities related to Barrett's esophagus. Furthermore, sera from all subjects were tested for the presence of *Helicobacter pylori* antibody. Subjects were asked to stop any acid inhibitory drugs or prokinetics 2 weeks before the examination. If, for any reason, subjects could not stop their medication, they were not included in the study. Furthermore, subjects with a previous history of upper gastrointestinal surgery were excluded from the study.

Barrett's esophagus was diagnosed on the basis of endoscopic detection of columnar epithelium extending continuously from the EGJ into the esophagus, without obtaining histological confirmation of the presence of intestinal metaplasia[18]. The EGJ was defined as the end of the palisading vessels of the lower esophagus[18]. Short segment Barrett's esophagus (SSBE) was defined as the presence of a columnar epithelium covering < 3 cm of at least one segment from the EGJ. Long segment Barrett's esophagus (LSBE) was defined as the presence of columnar epithelium > 3 cm from the EGJ and always covering the entire circumference[19].

Statistical analysis

All data are presented as the mean ± SD. The significance of differences among the control, SSBE and LSBE groups was assessed using the Mann-Whitney *U* test. $P < 0.05$ was considered significant.

RESULTS

Preliminary study evaluating manometric and endoscopic assessments of peristalsis and propulsive contractions, respectively

Physiological saline- and acid infusion-induced esophageal contractions were successfully recorded in five subjects using three different modalities simultaneously. Endoscopy revealed a total of 132 esophageal contractions without deglutition during physiological saline and acid infusion. Of these contractions, 74 (56%) and 58 (44%) contractions were determined to be propulsive and non-propulsive, respectively. All but one of the propulsive contractions assessed by endoscopy were recorded

Table 1 Clinical characteristics of the subjects in each group *n* (%)

	Control (*n* = 25)	SSBE (*n* = 16)	LSBE (*n* = 8)
Sex (male/female)	19/6	15/1[b]	8/0[b]
Age (yr)	32 ± 7	38.4 ± 14.4	38.0 ± 14.0
BMI (kg/m^2)	22 ± 2.8	22.7 ± 3.1	22.2 ± 3.5
No. smokers	7 (28)	4 (25)	4 (50)[b,d]
No. drinkers	20 (80)	14 (87.6)	7 (87.5)
No. *Helicobacter pylori* antibody positive	4 (18.2)	2 (12.5)	0 (0)[b,d]
No. with erosive reflux esophagitis	1 (4)	7 (43.8)[b]	5 (62.5)[b,d]

Data are expressed as absolute numbers (percentage) or mean ± SD. [b]P < 0.01 *vs* control; [d]P < 0.01 *vs* patients with short segment Barrett's esophagus (SSBE). LSBE: Long segment Barrett's esophagus; BMI: Body mass index.

Table 2 Results of the acid perfusion test in each group

	Lag time (s)	Intensity rate	APSS
Control group	6.1 ± 2.3	162 ± 150	9.0 ± 8.7
Barrett's esophagus	5.8 ± 2.2	162 ± 164	7.7 ± 6.1
SSBE	5.7 ± 2.3	199 ± 189	8.8 ± 6.9
LSBE	6.1 ± 2.0	88 ± 54	5.4 ± 3.3

Data are the mean ± SD. There were no significant differences between the groups in any of the parameters measured. Lag time is the time from the start of infusion until the onset of acid-induced symptoms, intensity rate refers to the intensity of the symptoms (scored over the range 1-10), and the acid perfusion sensitivity score (APSS) was calculated as the product of intensity rate and lag time. SSBE: Short segment Barrett's esophagus; LSBE: Long segment Barrett's esophagus.

as secondary peristaltic waves by manometry (Figure 2). Primary peristalsis by deglutition was not observed. It is likely that some sort of mechanical failure was responsible for the one propulsive wave showing no marked wave on manometry. Of the 58 non-propulsive contractions assessed by endoscopy, manometry recorded 51.7% and 48.3% as failed and no response, respectively. The high concordance between esophageal peristalsis assessed by manometry and propulsive contractions assessed by endoscopy indicates that endoscopic observation of propulsive contractions is a satisfactory method for evaluating secondary peristalsis induced by physiological saline and acid infusion.

Primary study evaluating acid-induced peristalsis and sensation in the esophagus

Twenty-six patients with Barrett's esophagus (mean age 40 ± 15 years) and 25 subjects undergoing endoscopic surveillance as part of their health check up (mean age 32 ± 7 years) were enrolled in the primary study. Of the 26 patients with Barrett's esophagus, 17 and nine were determined to have SSBE and LSBE, respectively. None of the 25 individuals undergoing the health check had Barrett's esophagus and so were used as the non-Barrett's esophagus control group. Two of the 26 patients with Barrett's esophagus (one in each of the LSBE and SSBE groups) were excluded from the study because there was unsatisfactory accumulation of solution following infusion in the esophagus, probably due to a large hiatal opening. The characteristics of the remaining subjects in each group are given in Table 1. Subjects in the non-Barrett's esophagus control group were younger, with a higher proportion of women, than patients with Barrett's esophagus. The rate of erosive reflux esophagitis was significantly higher in patients with Barrett's esophagus than in the control group.

Table 3 Frequency of primary and secondary contractions in the different groups following infusion of either physiological saline or acid

	No. primary contractions		No. secondary contractions	
	Physiological saline	Acid	Physiological saline	Acid
Control group	1.3 ± 3.3	0.3 ± 0.9	8.0 ± 2.8	8.4 ± 2.4
Barrett's esophagus	0.8 ± 1.2	0.7 ± 1.5	6.3 ± 3.1	7.2 ± 2.8
SSBE	1.1 ± 1.4	0.6 ± 1.0	7.3 ± 3.2	8.1 ± 2.9
LSBE	0.4 ± 0.7	1.0 ± 2.1	4.1 ± 1.2[b,d]	5.3 ± 1.2[b,d]

Data are the mean ± SD. Within the same infusion groups: [b]P < 0.01 *vs* control; [d]P < 0.01 *vs* patients with short segment Barrett's esophagus (SSBE). LSBE: Long segment Barrett's esophagus.

Esophageal sensation induced by acid infusion

Intensity rates following acid infusion were comparable between non-Barrett's esophagus controls and patients with Barrett's esophagus (Table 2). There were no significant differences between the groups in intensity rate. There was a tendency for lag time to be shorter in the LSBE compared with control group, although the difference did not reach statistical significance (88 ± 54 s *vs* 162 ± 150 s, respectively, P = 0.14). This may indicate that patients with LSBE were more sensitive to acid perfusion. Furthermore, there were no significant differences in the APSS following acid infusion between the non-Barrett's esophagus controls and patients with Barrett's esophagus. These results suggest that acid-induced esophageal sensations did not differ between patients with and without Barrett's esophagus.

Esophageal propulsive contractions

There were no significant differences among the groups in the frequency of primary contractions (Table 3) or in the frequency of secondary contractions in the esophagus following 10-min infusion of physiological saline or acid. However, when patients with Barrett's esophagus were divided into those with LSBE and SSBE, patients in the LSBE group exhibited significantly fewer secondary contractions following infusion of physiological saline than did the non-Barrett's esophagus controls and patients in the SSBE group (4.1 ± 1.2 *vs* 8.0 ± 2.8, P < 0.001 and 7.3 ± 3.2, P < 0.01, respectively; Table 3). Similarly, patients in the LSBE group exhibited a lower frequency of acid-induced secondary contractions than did either the control group or patients in the SSBE group (5.3 ± 1.2 *vs* 8.4 ± 2.4 and 8.1 ± 2.9, respectively, P < 0.01 for

both). Although there was a tendency for acid infusion to induce a higher frequency of secondary contractions than infusion of physiological saline, the differences did not reach statistical significance.

DISCUSSION

Endoscopy is essential for the diagnosis of mucosal and structural abnormalities of the upper gastrointestinal tract in individuals with GERD symptoms. The primary aim of endoscopic examination is to exclude malignant dysphagia and to detect Barrett's esophagus, with its associated cancer risk. In addition, Kawai *et al*[20] and we (present study) have demonstrated that transnasal endoscopy in unsedated patients can be used to evaluate esophageal function. Because esophageal propulsive contractions assessed by endoscopy corresponded well to esophageal peristalsis recorded by manometry, endoscopy-based testing, as described herein, may prove to be an adequate method with which to evaluate the frequency of acid perfusion-induced secondary peristalsis. Because this testing simultaneously assessed esophageal sensory status induced by acid infusion, we were able to obtain endoscopic findings and functional information for the esophagus in one examination.

Long-term exposure of the squamous mucosa of the lower esophagus to gastric acid or bile can lead to the development of Barrett's esophagus. Pathogenic exposure to refluxate can result from disturbances in anti-reflux barriers or delayed luminal acid clearance due to abnormalities in esophageal motility or the sensory system. Using endoscopy-based testing, we were able to demonstrate impairments in acid clearance due to a reduction in secondary peristalsis in patients with LSBE compared with patients with SSBE and the non-Barrett's esophagus controls. The impairment in secondary peristalsis revealed in the present study is comparable with the findings of a previous study, in which LSBE was characterized by a greater impairment in primary peristaltic wave amplitude than SSBE[21]. Primary peristalsis is the initial response to acid reflux in individuals when in the upright position, whereas secondary peristalsis is the initial clearing event when individuals are supine and asleep. Therefore, combined impairment in both primary and secondary esophageal peristalsis in patients with LSBE may elicit longer acid exposure. Indeed, it is known that the total percentage of time of esophageal acid exposure over 24 h is significantly greater in patients with LSBE compared with SSBE[22].

Impairment of secondary esophageal peristalsis has been reported in patients with various esophageal disorders other than Barrett's esophagus. For example, defective triggering of secondary peristalsis has been reported in patients with non-erosive reflux disease[23], whereas impaired esophageal bolus transit and clearance by secondary peristalsis was demonstrated in patients with non-obstructive dysphagia[24]. However, the mechanisms underlying defective secondary peristalsis remain unknown. Because secondary peristalsis is a reflex response to esophageal distention, the defect may lie in the esophageal motor nerves or muscles, esophageal sensation, the central integrative mechanisms, or a combination of these[16]. Iwakiri *et al* suggest that the primary defect lies in esophageal sensation because the triggering of secondary peristalsis was abnormal in all patients with normal primary peristalsis and, when triggered, the wave amplitude and velocity of the secondary peristaltic response were normal. Studies using esophageal balloon distention have reported decreased sensitivity to this stimulus in patients with Barrett's esophagus, and this could contribute to the impaired esophageal motility in Barrett's esophagus[25]. As indicated in Table 3, there was no difference in the frequency of secondary contractions induced by acid and saline. A possible explanation for this observation is that the effects of volume stimulation are greater than those of chemical stimulation in triggering secondary peristalsis, but the precise mechanisms involved remain unknown.

Previous studies have demonstrated that patients with Barrett's esophagus have impaired sensitivity to esophageal distention as well as visceral sensitivity to acid perfusion[25,26]. Using endoscopy-based testing in the present study, we tested visceral sensitivity to acid perfusion and showed that acid infusion-induced sensations in patients with LSBE were comparable to those in patients with SSBE and in the control group. This finding is not compatible with most previous studies that reported that patients with Barrett's esophagus have impaired visceral sensitivity to acid perfusion. It is possible that this discrepancy between the findings of the present and previous studies may be due to type I error, especially because of the small number of patients with LSBE in the present study. If we compared the lag time or APSS of patients with LSBE and the pooled values for each of these parameters in the control plus SSBE groups, the *P* values are 0.11 and 0.18, respectively. The mechanism underlying esophageal hyposensitivity in Barrett's esophagus has not been elucidated, but it has been proposed that Barrett's mucosa is less sensitive to chemical stimuli. For example, Fass *et al*[27] demonstrated increased sensitivity to acid in patients with Barrett's esophagus after complete reversal using multipolar electrocoagulation and suggested that chemoreceptor sensitization was possibly induced by the electrocoagulation technique. However, a recent study showed a significant decrease in esophageal chemoreceptor sensitivity to acid and alkaline in patients with successful reversal of Barrett's esophagus using argon plasma coagulation. Further studies are needed to evaluate esophageal hypersensitivities in Barrett's esophagus.

There are some limitations to the present study. First, the controls enrolled in the present study were younger than the patients with Barrett's esophagus, and the proportion of women in the control group was higher than that in the Barrett's esophagus group. To overcome this issue, we excluded women from the control and SSBE groups before analyzing the data. Although, after exclusion, the mean age of the control group remained lower than that of the Barrett's esophagus group (30.1 ± 5.2

years *vs* 38.1 ± 13.6 years, respectively), the results were very similar to those obtained using the original population. Aging may influence secondary esophageal propulsive contractions, and the older LSBE group may exhibit impaired peristalsis compared with the control group. However, differences in secondary esophageal propulsive contractions were seen between age-matched SSBE and LSBE groups, which suggests that, regardless of age, patients with LSBE have impaired secondary esophageal peristalsis at least in comparison with patients with SSBE.

The endoscopy-based testing used in the present study can evaluate the frequency of esophageal peristalsis but cannot provide detailed information regarding motility parameters, such as esophageal pressures; for detailed evaluation of esophageal dysmotility, examinations such as high-resolution manometry are necessary. Nevertheless, the endoscopy-based testing described in the present study can be used in clinical practice because of its simplicity and versatility.

In conclusion, observation of secondary peristalsis was possible using an endoscopic-based technique. Performing a simple acid perfusion study in unsedated LSBE patients, we were able to demonstrate disorders in secondary peristalsis using nasal endoscopy.

COMMENTS

Background

Because of its increasing prevalence in Asian countries, the clinical impact of gastroesophageal reflux disease (GERD) is also increasing. Furthermore, some of the patients with GERD will go on to develop Barrett's esophagus, which itself can progress to adenocarcinoma. The precise causative dysfunction varies between patients; thus, esophageal function tests are needed to determine the cause to enable cause-specific treatment. However, such examinations, including manometry, sensory testing and pH monitoring, are not routinely used in clinical practice because they are tedious, complicated, and invasive.

Research frontiers

The authors have developed a simple and versatile technique for esophageal examination based on transnasal endoscopy in unsedated patients. The test can simultaneously evaluate either structural abnormalities of the lumen and anti-reflux barriers or dysfunctions in esophageal peristalsis and sensation induced by acid infusion. Using this endoscopy-based test in the present study, the authors examined patients with Barrett's esophagus and healthy controls to identify abnormalities in esophageal function related to Barrett's esophagus.

Innovations and breakthroughs

First, manometric waves and esophageal contractions were evaluated in healthy subjects using three different modalities. An transnasal endoscopy, endoscopic ultrasonography and manometry was performed into the lower esophagus. Physiological saline was infused initially into the esophagus at a rate of 10 mL/min for 10 min. Subsequently, HCl was infused for 10 min at a similar rate. Esophageal contractions were analyzed. Next, using nasal endoscopy, esophageal sensations and contractions were investigated in patients with and controls without Barrett's esophagus following the same infusion method. The endoscopy-based testing used in the present study can evaluate the frequency of esophageal peristalsis. The endoscopy-based testing described in the present study can be used in clinical practice because of its simplicity and versatility.

Applications

The observation of secondary peristalsis was possible using an endoscopic-based technique. Performing a simple acid perfusion study in unsedated long segment Barrett's esophagus (LSBE) patients, we were able to demonstrate disorders in secondary peristalsis using nasal endoscopy.

Terminology

Primary and secondary peristalsis were defined as successful if a pressure wave > 12 mmHg at the two proximal esophageal recording sites above the lower esophageal sphincter and > 25 mmHg at the middle and distal esophageal recording sites progressively traversed all the esophageal recording sites. Peristaltic progression was defined as a peristaltic velocity of ≤ 6 cm/s. Barrett's esophagus was diagnosed on the basis of endoscopic detection of columnar epithelium extending continuously from the esophagogastric junction (EGJ) into the esophagus, without obtaining histological confirmation of the presence of intestinal metaplasia. The EGJ was defined as the end of the palisading vessels of the lower esophagus.

Peer review

This is a good descriptive study in which the authors analyzed dysfunctions in esophageal peristalsis and sensation in patients with Barrett's esophagus following acid infusion using endoscopy-based testing. The results are interesting and suggest that disorders in secondary peristalsis in patients with LSBE.

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META-ANALYSIS

Distance management of inflammatory bowel disease: Systematic review and meta-analysis

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Abstract

AIM: To review the effectiveness of distance management methods in the management of adult inflammatory bowel disease (IBD) patients.

METHODS: A systematic review and meta-analysis of randomized controlled trials comparing distance management and standard clinic follow-up in the management of adult IBD patients. Distance management intervention was defined as any remote management method in which there is a patient self-management component whereby the patient interacts remotely *via* a self-guided management program, electronic interface, or self-directs open access to clinic follow up. The search strategy included electronic databases (Medline, PubMed, CINAHL, The Cochrane Central Register of Controlled Trials, EMBASE, KTPlus, Web of Science, and SCOPUS), conference proceedings, and internet search for web publications. The primary outcome was the mean difference in quality of life, and the secondary outcomes included mean difference in relapse rate, clinic visit rate, and hospital admission rate. Study selection, data extraction, and risk of bias assessment were completed by two independent reviewers.

RESULTS: The search strategy identified a total of 4061 articles, but only 6 randomized controlled trials met the inclusion and exclusion criteria for the systematic review and meta-analysis. Three trials involved telemanagement, and three trials involved directed patient self-management and open access clinics. The total sample size was 1463 patients. There was a trend towards improved quality of life in distance management patients with an end IBDQ quality of life score being 7.28 (95%CI: -3.25-17.81) points higher than standard clinic follow-up. There was a significant decrease in the clinic visit rate among distance management patients mean difference -1.08 (95%CI: -1.60--0.55), but no significant change in relapse rate or hospital admission rate.

CONCLUSION: Distance management of IBD significantly decreases clinic visit utilization, but does not significantly affect relapse rates or hospital admission rates.

Key words: Telemanagement; Telehealth; Inflammatory bowel disease; Distance management; Self-management

Core tip: Distance management of inflammatory bowel disease (IBD) involves the use of telemedicine, web-based intervention, telephone clinics, patient directed open access clinics, and other methods that incorporate patient self-management strategies to manage patients remotely. This systematic review and meta-analysis of six randomized controlled trials shows that distance management of IBD significantly decreases clinic visit utilization, and can improve quality of life in certain groups. Consideration should be made in tailoring distance management approaches to select IBD patient populations.

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of inflammatory bowel disease: Systematic review and meta-analysis. *World J Gastroenterol* 2014; 20(3): 829-842 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i3/829.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i3.829

INTRODUCTION

Inflammatory bowel disease (IBD) is a group of chronic intestinal diseases that adversely affects quality of life and societal interaction and functioning. They are associated with significant morbidity and mortality. Patients have intermittent flares requiring adjustments in medications as well as frequent clinic visits, hospitalizations, and surgeries. In Canada, approximately 233000 Canadians have a diagnosis of IBD, and about 10200 Canadians are diagnosed each year[1]. The total direct costs of IBD are estimated at C$ 1.2 billion, with 76% being comprised of drug costs and inpatient hospitalizations. Total indirect costs are estimated to be C$ 1.6 billion, mainly due to lower labour participation rates[1]. With the increasing incidence rate of IBD over the past decade[1,2], there are longer wait lists to see specialists in clinics and increased health care resource utilization. The Canadian Association of Gastroenterology guidelines have recommended no longer than 2 wk waiting time for patients presenting with symptoms of active inflammatory bowel disease[3,4], yet in 2012 the average waiting time to see a consultant was 72 d[5]. This delay in medical assessment can adversely affect patients' quality of life[6]. This increasing burden of IBD on patients' quality of life and functioning and on national health care resources has been reported globally[7-10]. Earlier and more aggressive optimization of therapy could alter disease course and reduce hospitalization and health care resource costs[10,11].

Clinicians have focused on techniques to improve the out-patient management of IBD patients. Strategies to improve patient education alone increase IBD-related knowledge, but do not consistently improve clinical outcomes or decrease health care resource use[12-15]. Focusing on improving self-management behaviour, however, may be effective[16,17]. A previous systematic review on patient education and self-management reported that self-management strategies demonstrated improved outcomes of symptoms, psychological well-being, and health-care resource use[15].

In the past decade, clinicians have investigated using self-management as a component of distance management of chronic diseases. Telemedicine generally refers to "medicine practised at a distance"[18] and has been used to remotely manage several chronic diseases such as diabetes, heart failure, hypertension, chronic obstructive pulmonary disease with reported variable outcomes[18-20]. The main concept of medicine practised at a distance is that it incorporates a component of patient self-management where patients relay information about their state of health to a program or health care team, which gives them feedback. Patients can then adjust their therapy based on pre-determined algorithms or seek medical assessments. Researchers have recently investigated the use of telemedicine for IBD patients[21-24] with a recent review suggesting a potential role for telemedicine in the management of IBD patients[21].

Two prior reviews have looked at patient self-management and telemedicine management of IBD patients separately, and more recently studies of this topic have been published; therefore, the objective of this systematic review and meta-analysis is to provide an updated and comprehensive analysis of the efficacy of distance management methods *vs* standard clinic follow-up in the management of IBD patients. The primary outcome is the mean difference in quality of life, and secondary outcomes include the mean difference in relapse rate, clinic visit rate, and hospital admission rate.

MATERIALS AND METHODS

Registration of protocol

The protocol for this review was registered with Prospero, the international prospective register of systematic reviews in health and social care (registration number CRD42013004286) and can be found at the http://www.crd.york.ac.uk/prospero/ website.

Eligibility criteria

Randomized controlled trials (RCTs) comparing the efficacy of distance self-management *vs* standard clinic follow-up in the management of adult (> 16 years old) IBD patients were included. We had initially included cohort studies, before-after, and pilot studies in our search strategies, but as we aimed to present an analysis of the most stringent methodology, and there were sufficient RCTs, we excluded them from this review and meta-analysis.

Distance management intervention was defined as any remote management method in which there is a patient self-management component whereby the patient interacts remotely *via* a self-guided management program or electronic interface, and actively adjusts medications or self-directs open access to clinic follow up. Studies that included interventions that only involved improving patient education or managing stress and lifestyle, but had no self-management and no distance management component were excluded. The comparator group was standard clinic follow-up for the institution at the time of the study. Included studies had to implement the intervention and continue to follow the patient for at least 6 mo. Included studies had to report at least one of the outcomes of interest: quality of life (QoL), relapse rate, clinic visit rate, and hospital admission rate. Studies that did not report any of these outcomes were excluded from this review.

Search methods for identification of studies

A systematic search of the following electronic databases was performed in January 2013: Medline (1950-2013), PubMed (1950-2013), CINAHL (1937-2013), The Co-

chrane Central Register of Controlled Trials, EMBASE (1974-2013), KTPlus, Web of Science (1990-2013), and SCOPUS (1960-2013). We used the MeSH subject headings and text-words including "Inflammatory bowel disease", "crohn's", "ulcerative colitis", "patient education", "self-care", "self-management", "telehealth", "telemedicine", "ehealth", and similar keywords (Appendix A). If the database allowed, we exploded terms to be more inclusive. We also hand searched the conference proceedings of the major Gastroenterology and Inflammatory Bowel Disease conferences from 2008-2013 (Canadian Digestive Diseases Week, Digestive Diseases Week, Advances in IBD, and European Crohn's and Colitis Organisation congress). We searched for Internet publications using www.google.ca with the same search terms as for the electronic databases; and we also reviewed the reference lists of review articles and related studies. Searches were updated on a regular basis, and the last search completed on March 16, 2013.

Study selection

One reviewer (VH) completed the electronic search of the above listed databases, the hand searching of conference proceedings, and the internet search. Duplicate articles were removed using RefWorks 2.0 manager. VH screened the remaining titles to remove irrelevant articles, review articles, case series, and case reports. Using predetermined inclusion criteria, two reviewers (VH and KR) independently screened the remaining abstracts as "definitely include" (meeting all of the inclusion criteria, reported at least one of the outcomes), "maybe" (meeting some of the inclusion criteria, but unclear outcomes), and "definitely not" (did not meet any of the inclusion criteria). VH and KR then independently screened the full text manuscripts of the "definitely include" and "maybe articles", and excluded those that did not report any of the a priori outcomes. The decision on the final list of included articles was reached by discussion and consensus, with consensus on questionable inclusion or exclusion confirmed by a third reviewer (RF).

Data extraction

A data extraction form was developed based on the Cochrane data extraction form[25], and pilot tested for understanding and consistency among the two reviewers (VH and KR). Data regarding first author, publication date, study design, patient characteristics, intervention and control, and funding sources was extracted by VH and checked for accuracy by KR. VH and KR independently extracted outcomes data into the data extraction form. Disagreements were resolved by discussion and consensus. Attempts were made to contact study authors for data values and clarification of results.

Cross *et al*[26] expressed the method of analysis as intention to treat (ITT), yet the authors reported change from baseline IBDQ scores; the authors confirmed their results were calculated based on the final number of patients who completed the study [n = 14 Ulcerative colitis (UC) HAT and n = 18] best available care (BAC). The article by Elkjaer *et al*[27] reported on two parallel studies, one in Denmark and one in Ireland. Since each study population had separately randomized intervention and control groups, we felt it reasonable to treat these two studies as separate RCTs. They did not report values for the QoL score or the hospital admission rate. They also did not report SD for clinic visits, so SD was imputed from the P values. The articles by Kennedy *et al*[28] and Richardson *et al*[29] reported on different outcomes from the same RCT. The Robinson *et al*[30] study reported baseline IBDQ and end of study IBDQ but did not report SD, so we decided to use the largest SD from the Cross study, as both studies looked at change from baseline results. The Robinson *et al*[30] study did not report SD or P values for the relapse rate, so the largest SD of the studies (2.5 from Kennedy control group) was used. They also did not report SD values for clinic visits, so the SD was imputed from the p-value reported.

Quality and risk of bias assessment

Two reviewers (VH and KR) independently assessed each study for quality and risk of bias using the Cochrane Collaboration tool for risk of bias for RCT[31]. The final decision on overall risk of bias was reached through discussion and consensus.

Statistical analysis

All data was analyzed using Review Manager (RevMan) Version 5.2 (Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2012). Data was analyzed on an intention-to-treat basis, unless otherwise specified in the results of the articles, or in communications with the study's authors. Outcomes were all recorded as continuous variables, and the effect size was reported as mean difference with 95%CI. Pooled meta-analyses were completed on studies that reported the same outcomes. I^2 statistics were used to test for heterogeneity, and if there was significant heterogeneity, the random effects model was used. The source of heterogeneity was investigated by completion of subgroup analysis by type of distance method and disease type [UC *vs* UC and Crohn's disease (CD); there were no CD studies eligible for inclusion]. Sensitivity analysis was not done because of the significant heterogeneity among studies. Publication bias was planned if we had more than 10 studies, but it was not assessed because of the small number of studies.

RESULTS

Study selection

A total of 4061 articles were identified by the electronic search strategy (Figure 1), and 15 abstracts identified by hand searching conference proceedings. No additional articles were identified by the internet web search. After exact duplications were removed, 2884 articles remained. Screening by title excluded 2701 articles. Two reviewers independently screened the remaining 183 articles,

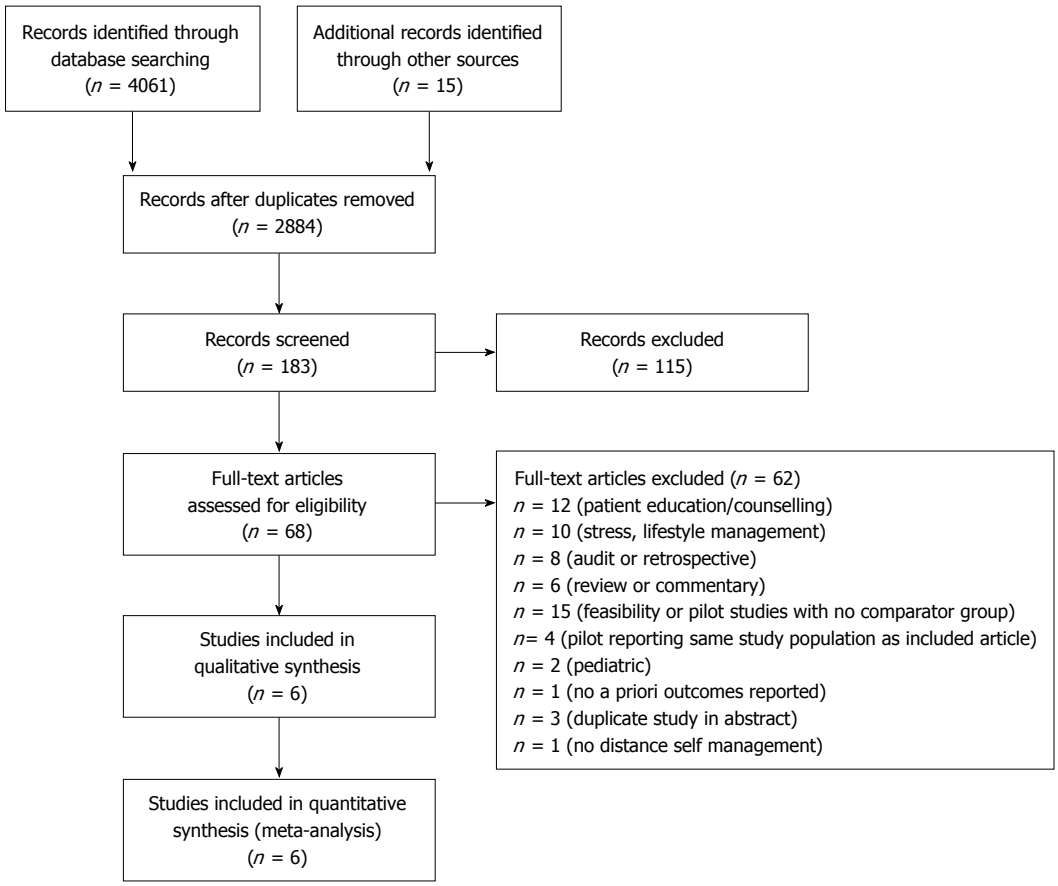

Figure 1 Flow chart of literature search results.

resulting in exclusion of 115 articles. Thus, 68 full text manuscripts were independently reviewed for eligibility. Of these, 62 were excluded for the follow reasons: (1) patient education/counseling only, no self-management *via* distance method[12-14,32-40]; (2) stress or lifestyle management, no self-management *via* distance method[41-50]; (3) audit or retrospective cohort study[51-58]; (4) review or commentary[17,59-63]; (5) feasibility or pilot studies with no comparator group[64-78]; (6) pilot study reporting on same study population as included study[24,79-81]; (7) pediatric patients[82,83]; (8) no a priori outcomes reported[84]; (9) duplicate studies[26,84-86]; and (10) smart phone method of symptom assessment, but no distance management or self-management[87]. Six full text articles reporting on six randomized controlled trials met the inclusion criteria for this systematic review[26-30,88]. An updated literature search was completed in March 2013, and did not reveal any new randomized controlled studies for this review.

Study characteristics

Table 1 summarizes the patient characteristics and interventions of the included RCTs. The Cross *et al*[26] RCT included UC patients from the University of Maryland, Baltimore and the Veterans Affairs, Maryland Health Care System, Baltimore. Patients were recruited through invitation by letter and also at the time of their clinic visits. Randomization by permuted block design with randomly varied block sizes was stratified by baseline disease activity strata, and assignment was concealed. However, post hoc analysis revealed that patients in the intervention group may have had higher disease activity, as they reported higher immunosuppressant use and lower baseline IBDQ scores. Research staff at study visits was blinded to treatment allocation. Patients answered questions weekly about symptoms, side effects, adherence, and received disease-specific education and customized action plans using the home unit, which then transmitted results to the decision support server. E-mail alerts were sent to the nurse coordinator if the patient met certain clinical conditions. The patients could also send electronic messages to the nurse coordinator, who then made management changes through consultation with the medical provider. The control group was managed by BAC.

The Elkjaer *et al*[27] article reported on two separate RCTs conducted on mild to moderate ulcerative colitis patients in Denmark (Herlev and Amager Hospital, Copenhagen) and Ireland (Adelaide and Meath Hopital in Dublin). Eligible patients who consented were randomized by a web-based randomization program, and assignments were concealed using closed, consecutive, numbered envelopes. They also included a historical control group from a separate hospital in Denmark, who were unaware of this trial, and prospectively from Adelaide

Table 1 Summary of included studies on distance management of inflammatory bowel disease in adults

Author, yr	Patients randomized/ baseline (N) intervention *vs* control	Disease Disease severity	Inclusion/exclusion	Mean age (yr) Control *vs* intervention	Male (%) Control *vs* intervention	Intervention	Control	Duration (mo)
Cross *et al*[26], 2012	47 pts rand. 14 web *vs* 18 BAC	UC	Not specified	40.3 *vs* 41.7	32 *vs* 40	UC HAT (Home telemanagement: - a home unit (laptop and electronic weight scale) a decision support server, -a web-based clinician portal	Best Available Care (educational material, action plan, clinics visits)	12
Elkjaer *et al*[27], 2010	233 pts rand. 105 web *vs* 106 control	UC mild/mod	Inclusion: age 18-69 yr, mild/moderate UC, treated with 5- ASA Exclusion: acute phase of co-morbid conditions, drug dependence or substance abuse, use of immunomodulators, frequent treatment with high dose systemic corticosteroids, likely requirement of IBD surgery, previous IBD surgery	40 *vs* 44 (P = 0.03)	49.5 *vs* 31.1 (P = 0.008)	Web-intervention (Educational training then www.constant-care.dk)	Conventional treatment and follow up in the IBD out-patient clinic	12
Elkjaer *et al*[27], 2010	100 pts rand. 51 web *vs* 41 control	UC	Same as above	41 *vs* 46	60.8 *vs* 41.5	Web-intervention (Educational training then www.constant-care.dk)	Conventional treatment and follow up in the IBD out-patient clinic	12
Kennedy *et al*[28], 2004 Richardson *et al*[29], 2006	700 pts rand. 270 interv. 365 control	Mild/mod CD (n = 231) UC or ID (n = 404)	Inclusion: UC or CD, over age of 16 yr, able to write English, attending a follow-up clinic Exclusion: Not specified	46.3 *vs* 44.4	43 *vs* 41.5	Guided self-management - patient guidebook - self-management plan - patient centered approach to care by a trained clinician - direct access to services for patients to self-refer	Management process deemed appropriate by the hospital specialist -6 sites follow long term - 2 sites discharge quiescent IBD -1 site no consistent follow up	12
Robinson *et al*[30], 2001	203 pts 101 interv. 102 control	UC	Inclusion: newly diagnosed Exclusion: require hospital outpatient follow-up for other illnesses, unable to read informed consent or follow written instructions	48 *vs* 49	48 *vs* 49	Personalised guided self-management regimen with direct access to outpatient care on request	Clinician's normal treatment and follow-up	Until 11 mo after last pt recruited
Williams *et al*[88], 2000	180 pts 88 interv. 92 control	CD (n = 78) UC or ID (n = 77) Proctitis (n = 25) Inactive or mildly active	Inclusion: over 18 yr, inactive or mildly active but stable IBD Exclusion: active disease requiring treatment, stoma, other disease requiring regular follow up, unable to comply with data collection	N/A (no significant difference reported)	N/A (no significant difference reported)	Open access follow up	Routine follow up	24

IBDQ: Inflammatory bowel disease questionnaire; SIBDQ: Short-IBDQ; NS: Not significant; ID: Indeterminate colitis; CD: Crohn's disease; UC: Ulcerative colitis.

hospital in Ireland. The intervention group received a 1.5 h disease specific presentation, and then a 1.5 h practical training session on the web-program http://www.constant-care.dk. Patients were instructed on how to recognize a relapse, and in case of relapse, would log on daily, complete the disease activity score, and follow management instructions, until they entered a green zone of quiescent disease. They then logged on weekly until 4 weeks after initiation of the relapse. Once in remission, they reported monthly. Patients could also email, call, or text the web-doctor. The control group continued to receive conventional treatment and follow up in the outpatient clinic.

The Kennedy *et al*[28]/Richardson *et al*[29] trial was a cluster-randomized multicentre trial conducted in the North West of England out of 7 teaching hospitals and 12 non-teaching hospitals. Both UC and CD patients were included, but specific inclusion and exclusion criteria were not reported. The first 38 eligible patients who consented were recruited at each site. The intervention consisted of four components (see Table 1). Those in the control group received management deemed appropriate by the hospitalist specialist.

The Robinson *et al*[30] RCT was conducted in four hospitals in the Greater Manchester area of the United Kingdom (Hope Hospital, Salford, Burgy General Hospital, Trafford General Hospital, and the Royal Oldham Hospital). Patients with ulcerative colitis were first assessed for suitability for inclusion by their normal clinician, and then interviewed by an investigator and invited to participate. Only patients in clinical remission were included in this study. The first 20 eligible patients who consented in each centre were included. Randomization was done by random number tables, and allocation was concealed by an assistant not involved in the study. The intervention consisted of a personalized guided self-management regimen, developed during a 15-30 min consultation by a clinician. Those in the control group received routine clinic follow-up.

The Williams *et al*[88] RCT was conducted out of two urban district general hospitals in Swansea (Morriston Hospital) and Neath (Neath Hospital), Wales. Patients with inactive or mildly active but stable disease were invited to participate. Patients with Crohn's disease, ulcerative colitis, indeterminate colitis, and proctitis were included. Those with ulcerative colitis or indeterminate colitis were analyzed as one group. The open access group contacted their general practitioner or the hospital about problems and were offered an early appointment.

Risk of bias within studies

The RCTs were of moderate to high risk of bias (Table 2). All of the studies reported on randomization and concealment, except for the Kennedy *et al*[28] and Richardson *et al*[29] trial. All of the trials were deemed high risk for performance bias because blinding could not be controlled for. Due to the nature of the intervention involving patient self-management, it was not possible to blind participants to the intervention. The outcomes were all affected by patient subjectivity, and therefore were prone to performance bias. There may have been changes to patient or physician behaviours depending on which group the patient was randomized to. The QoL outcome depended on self-reporting of symptoms. The relapse rate was somewhat dependent on the patient reporting symptoms compatible with a priori defined relapse/flare criteria. The number of clinic visits in the intervention group was dependent on the patient's responses to the self-management criteria, and their initiation to contact the health care team. Hospital admission rate was less likely prone to bias, as one would expect that admission to hospital was unlikely to be biased on group allocation.

There was attrition bias in the Cross *et al*[26] and Elkjaer *et al*[27] (Denmark) RCTs (see Table 2) with higher discontinuation or loss to follow up (LTF) in the intervention groups. In the Cross *et al*[26] study, trial completers had less extensive colitis than those who did not complete the trial. In the Kennedy *et al*[28]/Richardson *et al*[29] RCT, there was a bias towards greater loss to follow up in the control group; however the number of patients who did not complete the study was higher (12 *vs* 4) in the Morriston hospital.

There may also have been bias in terms of differences in patient characteristics. In the Cross *et al*[26] RCT, a significantly higher percentage (56%) of the UC HAT group were on immune suppressants compared to the BAC group (27%)[26]. In the Elkjaer *et al*[27] DenmarkRCT, there were significantly more males (49.5% *vs* 31.1%; $P = 0.008$) and younger patients (40-year-old *vs* 44-year-old; $P = 0.03$) in the web intervention group. There were no reported significant differences in baseline demographics in the Kennedy *et al*[28] and Richardson *et al*[29] trial, but they did not report specific inclusion/exclusion criteria. The Robinson *et al*[30] trial matched controls for age, sex, time since diagnosis, extent of disease, and numbers within hospitals. In the Williams *et al*[88] trial, there were no reported differences in baseline age, sex, diagnostic group, or quality of life. There may have been bias due to differences in standard clinic follow-up policies, as hospitals and clinicians have different follow-up protocols, as shown in Table 1.

Primary outcome: Quality of life

All five studies reported on QoL. There was a significant improvement in QoL ($P = 0.04$) in the Elkjaer *et al*[27] Denmark web group, but no significant difference in QoL in the Ireland web group. They did not report the actual QoL score values, thus their results could not be pooled. The Williams *et al*[88] trial reported the various components of the UK Inflammatory Bowel Disease questionnaire and found no significant change in mean health related QOL scores; although, there was some deterioration in both groups on most subscales. They did not report summary values that could be pooled. Three trials[26,28,30] ($n = 338$) reported changes in IBDQ scores after one year of intervention and were included in the

Table 2 Risk of bias of included studies on distance management compared with standard clinic follow-up for adult inflammatory bowel disease patients

Author, yr	Selection bias	Performance bias	Detection bias	Attrition bias	Reporting bias	Source of funding
Cross *et al*[26], 2012	Low (random permuted block design; concealed)	High	Low (research staff blinded to treatment allocation)	High (more discontinued in intervention group 8/25 *vs* control 1/22)	Low	Broad Medical Research Program, University of Maryland General Clinical Research Center Grant, General Clinical Research Centers Program, NCRR, NIH, Baltimore Education and Research Foundation
Elkjaer *et al*[27], 2010	Low (randomisation program; closed envelope)	High	High	High (LTF higher in the web group 24% *vs* control 17%)	Low	Colitis Crohn Patient Organization, Moran' s Foundation, Vibeke Binder and Povl Riis' Foundation, Bayer Health Care Funding, Augustinus Foundtaion, Munkholms Foundation, Tillotts Funding, Scientific Councel at Herlev Hospital, Prof. Fagerhol Research Foundation, Aase and Einar Danielsen Foundation, Ole Trock-Jansen and Hustrus Foundation, and European Crohn Colitis Organization
Elkjaer *et al*[27], 2010	Unclear (cluster randomization; no mention of concealment)	High	High	High (LTF higher in control group)	Low	
Kennedy *et al*[28], 2004 Richardson *et al*[29], 2006	Low (random number tables; concealed)	High	High	High (LTF higher in control group 24% *vs* intervention 13%)	Low	Health Technology Assessment Programme of the United Kingdom NHS (MS) Career Scientist Award in Public Health, NHS R and D (GS) Researcher Development Award, NHS R and D
Robinson *et al*[30], 2001	Low (computer generated lists, concealed)	High	High	Low	Low	(AR) United Kingdom Medical Research Council Training Fellowship
Williams *et al*[88], 2000	Low (computer generated lists, concealed)	High	High	Low	Low	NHS research and development primary/ secondary care interface programme, West Wales and Swansea Group of the National Association for Colitis and Crohn's Disease.

LTF: Lost to follow-up; NCCR: National Center for Research Resources; NIH: National Institute of Health; NHS R and D: National Health Services Research and Development.

meta-analysis. The baseline IBDQ scores in the Cross *et al*[26] and Robinson *et al*[30] trials differed between groups; thus for the meta-analysis, we used their published change in IBDQ scores, adjusted for baseline IBDQ scores. The Kennedy *et al*[28] trial presented data that was already adjusted for baseline scores.

Effect sizes varied, and there was significant heterogeneity (I^2 = 96%; P < 0.00001). Subgroup analysis by type of distance management (Figure 2A) decreased the heterogeneity, and also showed that the electronic telemanagement system significantly improved QoL mean difference 16.30; 95%CI: 12.36-20.24). Subgroup analysis by disease type (UC *vs* both) did not decrease heterogeneity within groups and did not result in significant mean differences (Figure 2B).

Secondary outcome: Relapse rate

Four RCTs[27,28,30] (n = 450) reported relapse rate. Effect sizes varied, however, there was significant heterogeneity (I^2 = 75%; P = 0.0007). Subgroup analysis by type of distance management (Figure 3A) decreased heterogeneity. It also showed that the Elkjaer *et al*[27] trials, which used the electronic, web-based distance management, favoured the control group mean difference 0.33; 95%CI: 0.15-0.51, while the patient directed open access clinic management studies favoured the distance management group mean difference -0.40; 95%CI: -0.77--0.03. Heterogeneity may also be explained by differences in disease type (Figure 3B), as four RCTs[26,27,30] included only UC patients, while the Kennedy *et al*[28]/Richardson *et al*[29] and the Williams *et al*[88] RCT included UC and CD. The UC studies were in favour of control mean difference 0.25; 95%CI: -0.04-0.54), while the UC and CD study was in favour of distance management mean difference -0.40; 95%CI: (-0.83-0.03); neither of the effect sizes were statistically significant (Figure 3B).

Secondary outcome: Number of clinic visits/patient/year

Five RCTs[27,29,30,88] reported on the number of outpatient clinic visits. The Williams *et al*[88] data could not be pooled with the others, as they reported clinic visits over 24 mo; however results favoured the distance management group 4.12 (SD 3.41) visits per patient *vs* 4.64 (SD 2.38) visits per patient in the control group). Effect sizes varied, and there was significant heterogeneity (I^2 = 89%; P < 0.00001), but even with subgroup analysis by interven-

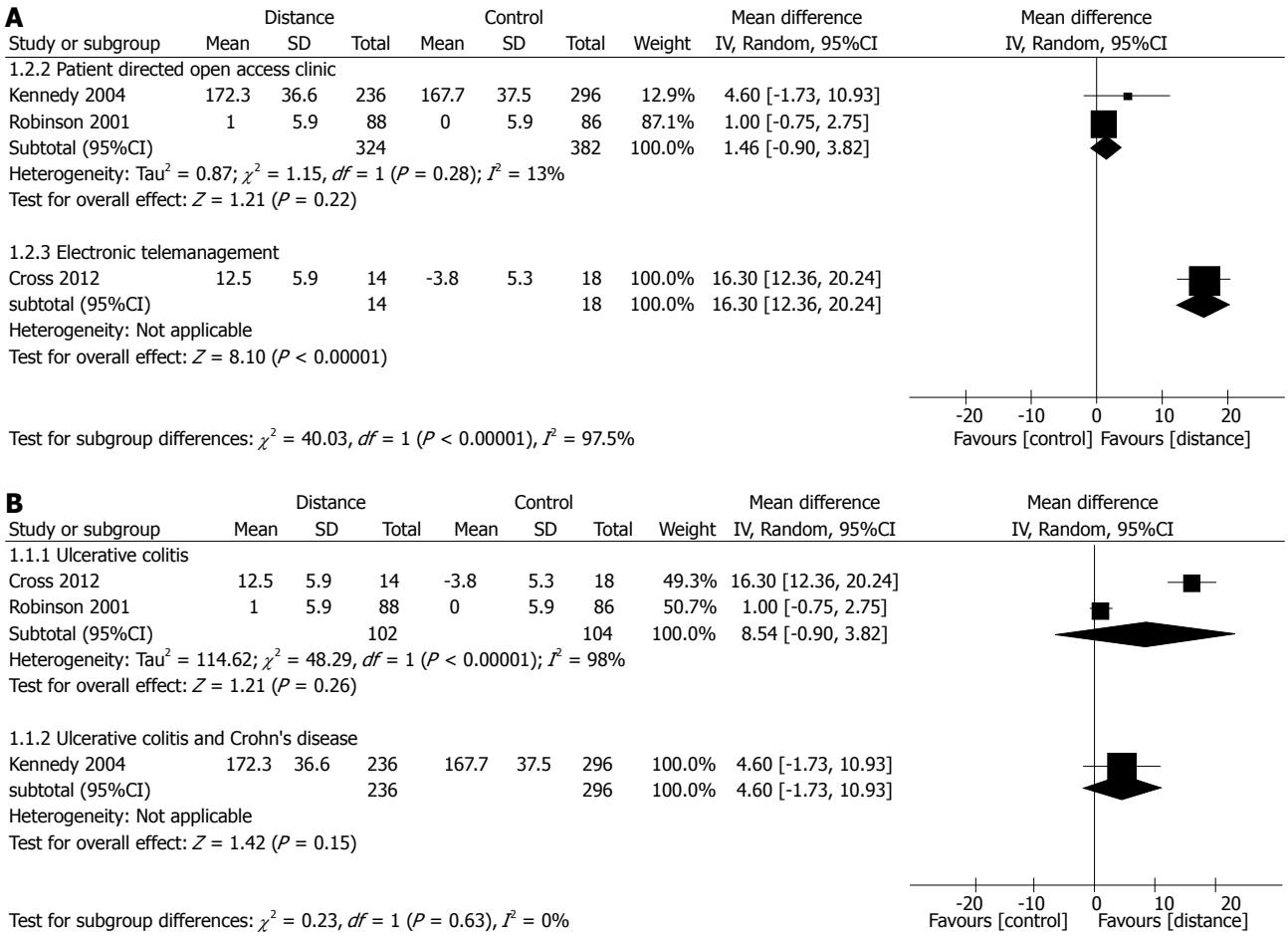

Figure 2 Mean change in quality of life between distance management and standard clinic follow up subgroup analysis by intervention and disease. A: Intervention; B: Disease.

tion type (Figure 4A) and disease (Figure 4B), the results favoured distance management.

Secondary outcome: Hospital admission rate

We had a priori planned to analyze the number of hospital admissions/patient/year, but only the Elkjaer *et al*[27] trials reported non-significant differences but did not provide actual values for this outcome. Two trials[29,88] reported mean number of hospital inpatient days but could not be pooled as one trial duration was 24 mo compared to 12 mo. However, there were no significant differences in the number of inpatient days per patient over 24 months reported in the Williams *et al*[88] open-access intervention trial [open access 0.83 (SD 3.53) *vs* control 0.41 (SD 1.74); $P = 0.71$] or over 12 mo reported in the Kennedy *et al*[28]/Richardson *et al*[29] trial [self-care 1.01 (SE 0.36) *vs* control 1.18 (SE 0.28); NS]. Thus, although actual data values are not available, there was no significant difference reported in hospital admission rate between distance management and standard clinic follow-up.

Publication bias

Our search strategy was a comprehensive search which included conference proceedings and internet searches for unpublished studies. We were unable to do a funnel plot to assess for publication bias, due to the small number of eligible studies. However, the results reported from the included RCTs were equivocal in favouring distance management *vs* standard clinic follow-up, so it does not appear that there is publication bias in this field of interest.

DISCUSSION

This review included six randomized controlled trials comparing distance management and standard clinic follow-up of inflammatory bowel disease patients for a total 1463 randomized IBD patients[26-30,88]. Three trials used electronic telemanagement or web-based systems, and three trials used patient directed open-access clinics. Distance management of a chronic disease such as IBD ideally would maintain or improve QoL, maintain or decrease relapse rates, and decrease health care utilization. This review shows that distance management intervention resulted in variable improvements in QoL, clinic visits, relapse rates, and hospitalization rates. Overall, the results support the rationale of using distance management in the management of IBD patients.

The six RCTs showed a trend toward an improvement in QoL scores overall[26-30,88]. Subgroup analysis showed that the UC Home telemanagement system resulted in

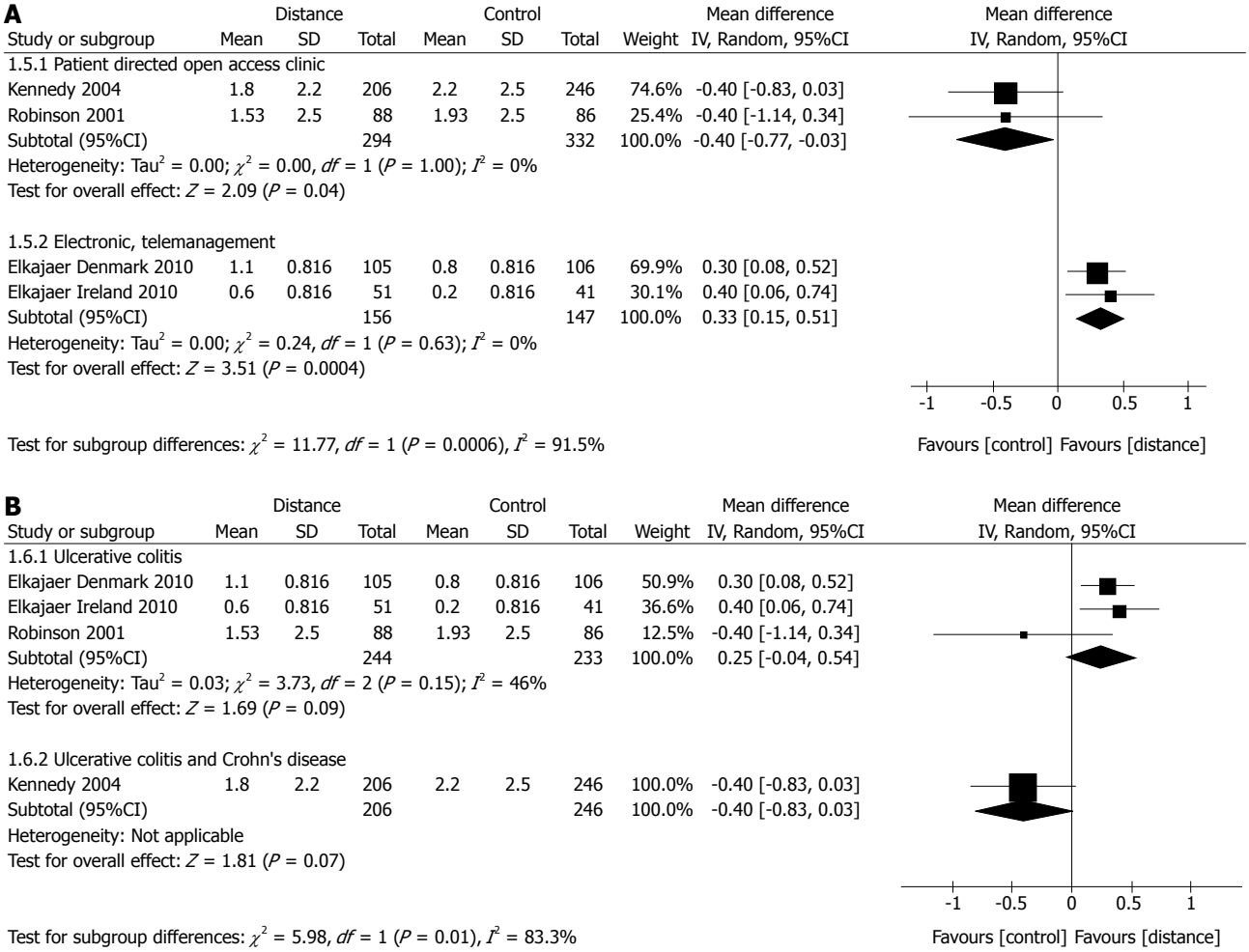

Figure 3 Difference in relapse rate between distance management and standard clinic follow-up subgroup analysis by intervention and disease. A: Intervention; B: Disease.

significantly improved QoL scores (mean difference 16.30) while the patient-directed open-access clinic intervention resulted in a non-statistically significant improvement in QoL. One potential reason for this difference is there is more interaction with the home telemanagement system. The patient answers specific questions, receives instructions, and is able to email the clinicians. On the contrary, in the open-access clinic approach, the patient is left to self-direct their own management based on their symptoms or a pre-determined management plan. Another potential reason may be that patients in the Cross *et al*[26] UC home telemanagement system group had higher immunosuppressant use and lower baseline IBDQ scores, and thus may have had higher disease activity than the control group. This may have resulted in significance in even small improvements in QoL. However, any small, even if not statistically significant, improvement in QoL may be clinically important and beneficial to IBD patients. Thus, even the open-access clinic approach may be useful in improving IBD patient QoL.

All six RCTs showed a significant decrease in clinic utilization in the intervention group, regardless of type of intervention or disease type[26-30,88]. On average, the interventions decreased the number of clinic visits from 2 to 3 visits to 1 visit per patient per year. This might allow these clinic visit times to become available for other patients or urgent cases. This may help consultants to achieve the target CAG waiting time of 2 wk for patients presenting with symptoms of active inflammatory bowel disease. However, telemedicine still requires time from the nursing staff or the physician, and exchange of clinic visits for telemedicine contact and follow up may still result in equivalent use of health care resources.

Since decreased clinic visit utilization may theoretically affect relapse rates and hospitalization rates, this meta-analysis also looked at these two outcomes. The Elkjaer *et al*[27] RCTs reported increased relapse rates in the web-based group, thus favouring the control group. On the other hand, the patient directed open access studies favoured the intervention group. This difference could be explained by a difference in the definition of relapse. The Elkjaer *et al*[27] trials used an objective measure of SCCAI (Simple Clinical Colitis Activity Index) score > 5, and the Kennedy *et al*[28]/Richardson *et al*[29] and Robinson *et al*[30] trials used patient self-reported relapses. However, the absolute difference in relapse rate was small -0.40 (patient

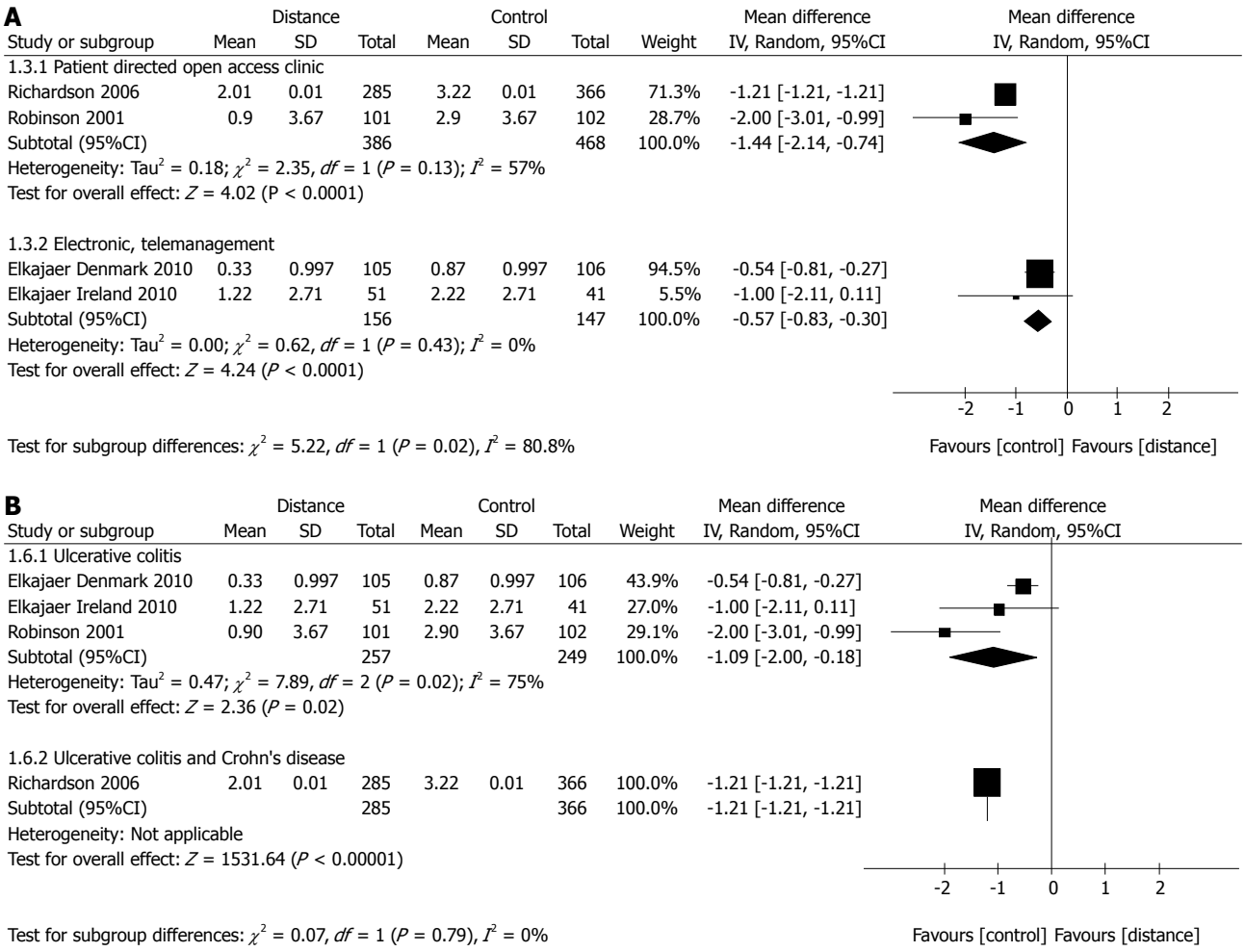

Figure 4 Difference in number of clinic visits per patient per year between distance management and standard clinic follow-up subgroup analysis by intervention and disease. A: Intervention; B: Disease.

directed open access clinic) *vs* 0.33 (web-based) relapse per year per patient. This difference in relapse rate may not be clinically significant, since there was no difference in hospital admission rate between intervention and control groups, and there was actually a decrease in the number of clinic visits per patient per year.

There are several limitations of this review. The overall risk of bias of the included studies was moderate to high when assessed using the Cochrane Collaboration risk of bias tool. Reasons included inability to blind participants due to the nature of the intervention of interest and unequal loss to follow-up and study completion rates between groups. Since some studies did not fully report inclusion/exclusion criteria, there may have been subtle differences in the study populations that may have led to some of the reported differences in effect size. There may be differences in the personalities of patients who consented to participate, in terms of their perceptions of self-reported disease relapse[89] or their beliefs about personal control and self-management[90]. In addition, some of the studies did not clarify specific inclusion and exclusion data, and therefore there may have been differences in disease activity or severity between groups. This may have affected quality of life scores or number of clinic visits and presentations to hospital for admission. In addition, these types of management methods for any chronic disease may be more beneficial for select patients at certain disease stages[17] or patients living farther away from urban centres. Further studies comparing different management strategies for patients with different disease severities would be useful, as patients with more severe or active disease may require more intensive management.

Another limitation was heterogeneity in the type of distance management and in the reported standard clinic follow-up policies between different hospitals. This made it difficult to pool results from different studies; however, sub-group analysis showed some differences between web-based and patient directed open access interventions. Finally, there was an issue with variable and incomplete data-reporting. Some results could not be pooled as raw data was unavailable. Standard deviation had to be imputed for many of the variables. However, overall, subgroup analysis by intervention or disease type showed consistent mean differences.

This review only included detailed analysis of randomized controlled trials of distance management of

IBD. However, non-randomized studies have shown benefit with telephone clinics[32,53,54,91], nurse specialist management[37,38,55,58], e-mail[75,92], smart-phone programs[76,77,89,93], chronic care models[47-51], and virtual clinics[68]. Incorporating these distance management methods may also be useful in improving standard clinical care and should be considered for future randomized controlled trials.

In conclusion, distance management of IBD decreases clinic visit utilization, but it does not significantly improve patients' quality of life, relapse rates, or hospital admission rates. Consideration should be made in tailoring these approaches to select patient populations. Perhaps a combined web-based and patient directed open access clinic distance management program, whereby patients interact with an electronic web-based management program and are able to initiate self-treatment strategies and self-referral to clinic assessments, may be the solution. Further studies are needed to determine the best type and the cost effectiveness of distance management of inflammatory bowel disease patients. Future randomized controlled trials comparing different types of distance management with different groups of IBD patients may help to determine which type of distance management is the optimal method for specific groups of patients.

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COMMENTS

Background

Inflammatory bowel disease is a group of chronic bowel diseases that affect patients of all ages, and all locations. The health care utilization of inflammatory bowel disease patients is increasing over time. Distance management of patients using remote methods that involve self-management strategies and patient-health care provider interaction, may be a mechanism to improve management of inflammatory bowel disease. Distance management of inflammatory bowel disease includes methods such as internet and web-based programs, telephone clinics, digital phones, patient self-management *via* pre-determined action plans, patient self-directed open access clinics.

Research frontiers

Prior reviews have looked at self-management and telemedicine strategies separately. This systematic review and meta-analysis examines the effectiveness of distance management using patient self-management techniques in improving quality of life, clinic visit utilization, relapse rate, and hospitalization rate in IBD patients.

Innovations and breakthroughs

This review and meta-analysis presents a unique comparison of distance management methods to standard clinical care of IBD patients. The authors have found that distance management methods significantly decreases clinic visit rates, and slightly improves quality of life in inflammatory bowel disease (IBD) patients, but does not significantly affect the relapse rate or hospital admission rate.

Applications

Distance management of IBD can be an important part of the management of IBD patients, but may require tailoring of these approaches to select patient populations. A combined web-based and patient directed open access clinic distance management program, whereby patients interact with an electronic web-based management program and are able to initiate self-treatment strategies and self-referral to clinic assessments, may be a solution.

Terminology

The main concept of distance medicine is that it incorporates a component of patient self-management where patients relay information about their state of health to a program or health care team, which gives them feedback. Patients can then adjust their therapy based on pre-determined algorithms or seek medical assessments.

Peer review

This is an interesting and well-written systematic review and meta-analysis on a relevant and current topic. The question of whether IBD patient self-management can be optimized through "distance" techniques is a worthy topic for consideration. This paper is well written and the methodology for the most part was spot-on.

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META-ANALYSIS

Remains of the day: Biliary complications related to single-port laparoscopic cholecystectomy

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Abstract

AIM: To assesse the rate of bile duct injuries (BDI) and overall biliary complications during single-port laparoscopic cholecystectomy (SPLC) compared to conventional laparoscopic cholecystectomy (CLC).

METHODS: SPLC has recently been proposed as an innovative surgical approach for gallbladder surgery. So far, its safety with respect to bile duct injuries has not been specifically evaluated. A systematic review of the literature published between January 1990 and November 2012 was performed. Randomized controlled trials (RCT) comparing SPLC versus CLC reporting BDI rate and overall biliary complications were included. The quality of RCT was assessed using the Jadad score. Analysis was made by performing a meta-analysis, using Review Manager 5.2. This study was based on the Preferred Reporting Items for Systematic reviews and Meta-Analyses guidelines. A retrospective study including all retrospective reports on SPLC was also performed alongside.

RESULTS: From 496 publications, 11 RCT including 898 patients were selected for meta-analysis. No studies were rated as high quality (Jadad score $\geqslant$ 4). Operative indications included benign gallbladder disease operated in an elective setting in all studies, excluding all emergency cases and acute cholecystitis. The median follow-up was 1 mo (range 0.03-18 mo). The incidence of BDI was 0.4% for SPLC and 0% for CLC; the difference was not statistically different (P = 0.36). The incidence of overall biliary complication was 1.6% for SPLC and 0.5% for CLC, the difference did not reached statistically significance (P = 0.21, 95%CI: 0.66-15). Sixty non-randomized trials including 3599 patients were also analysed. The incidence of BDI reported then was 0.7%.

CONCLUSION: The safety of SPLC cannot be assumed, based on the current evidence. Hence, this new technology cannot be recommended as standard technique for laparoscopic cholecystectomy.

Key words: Bile ducts; Cholecystectomy; Single port; Single incision

Core tip: This study assessed the rate of Bile Duct Injuries and overall biliary complications during single port laparoscopic cholecystectomy. A systematic review of the literature was performed, including 11 randomized controlled trials (898 patients) and 60 non-randomized trials (3599 patients). No statistically significant differences were found. However, interpretation of the results was impaired by several limitations. Based on a retrospective analysis, an incidence of bile duct injuries up to 0.7% was found. The safety of single-port laparoscopic cholecystectomy cannot be assumed, based on the current evidence. Hence, this new technology cannot be recommended as standard technique for laparoscopic cholecystectomy so far.

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INTRODUCTION

The recent decade has seen striking technical changes in gastrointestinal surgery. Surgeons' interest and expectations were high while waiting for the next technical evolution after laparoscopy[1]. Some of these expectations were also fuelled by industry and by patients themselves.

Traditionally, many innovations in digestive surgery were first evaluated by using cholecystectomies in humans as an *in vivo* model. The large number of patients in good condition, the non-reconstructive nature of such an intervention, and the availability of a standardized operative technique for comparison are the main characteristics predisposing gallbladder surgery to assess new technologies. Complication rates are generally low, and the only real difficulty is to avoid bile duct injuries (BDI)[2]. Indeed, BDI represent the key criteria comparing a novel technique to the current standard laparoscopic approach. The widespread implementation of conventional laparoscopic cholecystectomy (CLC) in 1990's was associated with an increased rate of bile duct injuries[3]. The lessons learnt by the clinical introduction of laparoscopy need to be re-considered since several novel techniques of minimal invasive surgery are on the point of being introduced as routine procedures in surgery without enough evidence on safety issues.

An increasingly voluminous literature on single port laparoscopic cholecystectomy (SPLC) has become available after five years of intense publications worldwide. In their meta-analysis, neither Sajid *et al*[4] nor Trastulli *et al*[5] found statistical significant differences between the two procedures in term of complications. However, BDI injuries were not analyzed specifically. On the other hand, Joseph *et al*[6] reported BDI rate as high as 0.72%, based on retrospective data.

The aim of this systematic review was to evaluate the current rate of BDI and overall biliary complication rate during single port laparoscopic cholecystectomy and to compare it with conventional laparoscopic cholecystectomy.

MATERIALS AND METHODS

Study selection

A systematic review of the literature published between January 1990 and November 2012 was performed by the first author from Medline, Embase and Web of Science databases. This search was then cross-checked by the senior author (Schäfer M). Following Medical Subject Heading (MeSH) and non-MeSH keywords were used: "cholecystectomy", "laparoscopy", "single trocar", "single port", "single incision", "single site", "single access", "trans-umbilical", "single incision laparoscopic surgery", "laparoendoscopic single site". The search was also enlarged using the "related articles" function and by manual cross-check of individual articles. A flowchart of the selection process, according to Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) statements[7], is presented in Figure 1.

Inclusion/exclusion criteria

Initially, all clinical trials conducted on humans and reporting clearly surgical complications were included. There were no language limitations. A second step of the analysis included only randomized controlled trials. We excluded all papers not related to the subject, all series that did not report surgical complications and all series including less than 10 patients.

Outcome of interest

The primary endpoint was the rate of BDI, as described by Strasberg *et al*[2]. Secondary endpoints were the rate of overall biliary complications, defined as any complication related to the biliary system that required the readmission of patients or additional interventions, such as endoscopic retrograde cholangiopancreatography or percutaneous drainage of bilioma. The time of follow-up, the realization of intraoperative cholangiography (IOC) and the report of a true critical view were also considered.

Study quality

The quality of randomized controlled trials (RCT) was assessed using the Jadad score[8]. Studies with a score of $\geqslant 4$ were considered high quality studies. Two reviewers (Allemann P, Schäfer M) evaluated independently all RCT included in the analysis. Results were compared thereafter and consensus were established when discrepancies were found.

Statistical analysis

Meta-analysis was conducted according to the PRISMA guideline. Investigations were performed using Review Manager 5.2 (release November 8, 2012. Cochrane Information Management System). Evaluating dichotomous variables, OR were used in the statistical analysis and favored conventional laparoscopic cholecystectomy if < 1. A statistically significant result was considered if the P value was < 0.05, and if the confidence interval did not include the value 1. Heterogeneity was assed using χ^2 and I^2 statistics. High I^2 and P value < 0.001 indicated significant heterogeneity between the studies. A power calculation was made using STATA 12.0 (Stata Corp., College Station, TX, United States), with an alpha-error of 0.05 (two sided) and a power of 80%.

RESULTS

After electronic research and manual cross match, 496 abstracts were collected. From these, 71 studies were included in the final analysis. Eleven studies were random-

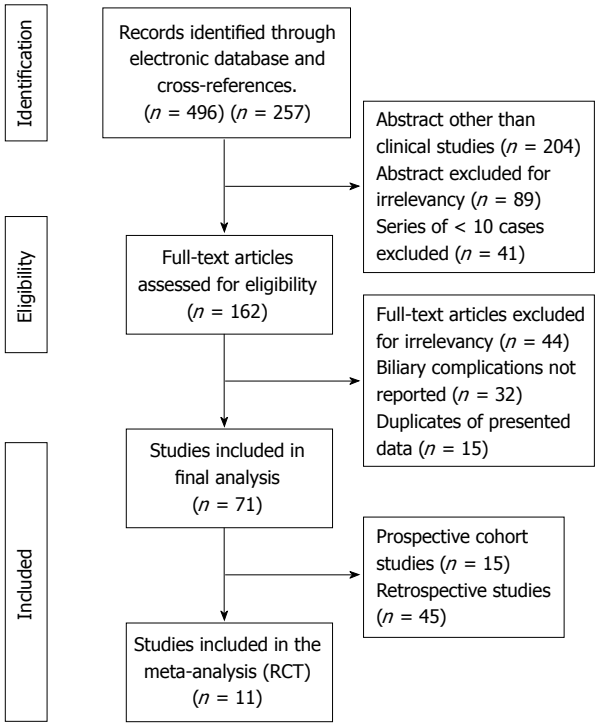

Figure 1 Flowchart diagram of the systematic review. RCT: Randomized controlled trials

ized controlled trials, including a total of 839 patients (438 SPLC, 401 CLC)[9-19]. Eight series were single center[9,12,14-19], whereas the three remaining trials were multicenter studies[10,11,13]. Four studies have been performed in Asia[9,12,15,18], four in Europe[11,14,16,19], one in North America[17], one in South America[10], and one in Europe and North America[13]. Conventional laparoscopic cholecystectomy was performed with four ports in nine series[9-11,13-15,17-19] and three ports in two[12,16]. Operative indications included benign gallbladder disease operated in an elective setting in all studies, excluding all emergency cases and acute cholecystitis. Body mass index > 30 kg/m^2 was considered as a contra-indication in five studies[11-13,16,19], > 40 kg/m^2 in one study[17] and previous upper-GI surgery in eight studies[9,11-13,15,16,18,19]. Repartition of the studies according to Jadad score is presented in Figure 2. No studies were rated as high quality (≥ 4 points). The median follow-up was 1 mo (range 0.03-18 mo).

The incidence of BDI was 0.4% for SPLC, compared to 0% for CLC (OR = 4.5), but the difference was not statistically different (P = 0.36, 95%CI: 0.22-96). The heterogeneity was zero (χ^2 = 0.00, df = 1, P = 0.95; I^2 = 0%). Of note, only two studies including 148 patients contributed to the analysis, while the remaining nine studies were not included because no events were observed in both groups. Forrest plots are presented in Figure 3A.

The incidence of overall biliary complication was also higher for SPLC compared to CLC with 1.6% *vs* 0.5% (OR = 3.2), but again, the difference did not reached statistically significance (P = 0.21, 95%CI: 0.66-15). The heterogeneity was zero (χ^2 = 0.84; I^2 = 0%). Six studies including 482 patients contributed to the analysis.

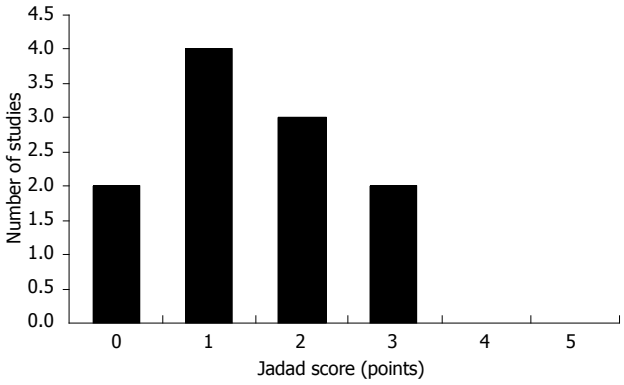

Figure 2 Repartition of the studies according to Jadad score.

Five studies were not included because no events were observed in both groups. Forrest plots are presented in Figure 3B.

Three of 11 studies reported a perioperative assessment of the critical view of safety, for a total of 119 patients. The use of intraoperative cholangiography was also described in three studies for a total of 77 patients. The procedure was performed in 100%, 70% and 5% of SPLC patients, respectively.

For the non-randomized reports, 60 studies were included with a total of 3599 patients[20-79]. Fifteen were prospective cohort studies and 45 were purely retrospective. From this survey, 25 BDI were reported. The BDI rate was 0.7% and the overall biliary complication rate was 2%. The distribution of BDI according to the Strasberg classification[2] is presented in Figure 4; 12/25 cases of BDI were type-A injuries, and 5/25 were reported, but not specified otherwise. The overall surgical complication rate was 5%. One third (62/180) of them were related to the surgical site (seroma, hematoma, infection, hernia). In particular, 18 postoperative hernias were reported (overall hernia rate of 0.5%). The median follow-up of patients was 2 mo (range 0.03-24 mo) (Table 1).

DISCUSSION

This meta-analysis assessed the risk of BDI and other biliary complications of single port access cholecystectomy; and compared it to conventional laparoscopic cholecystectomy. The analysis of the current literature revealed an increased rate for BDI of 0.4% and other biliary complications (1.6%) compared to conventional laparoscopic cholecystectomy (0%, 0.5% respectively). However, the differences found were not statistically significant.

Cautious interpretation is mandatory since many limitations impact on these results. Only two series contributed to the analysis of the BDI rate[11,12] and this is strictly not enough to build a statistically valid analysis. Moreover, the quality of these RCT was low, as Jadad scores were not above three, as illustrated in Figure 2. For example, still many investigators use envelope-based randomization technique, and/or blinding is not systematically performed. As stated by Baum[80], overcoming the

A

Study or subgroup	SPLC Events	SPLC Total	CLC Events	CLC Total	Weight	Odds ratio M-H, Fixed, 95%CI
Madureira 2012	0	28	0	29		Not estimable
Ma 2011	0	21	0	22		Not estimable
Aprea 2011	0	25	0	25		Not estimable
Tsimmoyiannis 2010	0	20	0	20		Not estimable
Asakuma 2011	0	24	0	25		Not estimable
Lai 2011	0	24	0	27		Not estimable
Bucher 2011	0	75	0	75		Not estimable
Sasaki 2012	0	27	0	27		Not estimable
Phillips 2011	0	117	0	80		Not estimable
Lirici 2011	1	20	0	20	47.5%	3.15 [0.12, 82.16]
Cao 2011	1	57	0	51	52.5%	2.73 [0.11, 68.63]
Total (95%CI)		438		401	100.0%	2.93 [0.30, 29.03]
Total events	2		0			

Heterogeneity: $\chi^2 = 0.00$, $df = 1$ ($P = 0.95$); $I^2 = 0\%$

Test for overall effect: $Z = 0.92$ ($P = 0.36$)

Odds ratio M-H, Fixed, 95%CI

0.001 0.1 1 10 1000

Favours SPLC Favours CLC

B

Study or subgroup	SPLC Events	SPLC Total	CLC Events	CLC Total	Weight	Odds ratio M-H, Fixed, 95%CI
Asakuma 2011	0	24	0	25		Not estimable
Aprea 2011	0	25	0	25		Not estimable
Lai 2011	0	24	0	27		Not estimable
Bucher 2011	0	75	0	75		Not estimable
Madureira 2012	0	28	0	29		Not estimable
Ma 2011	1	21	0	22	11.4%	3.29 [0.13, 85.44]
Lirici 2011	1	20	0	20	11.7%	3.15 [0.12, 82.16]
Sasaki 2012	1	27	0	27	11.9%	3.11 [0.12, 79.87]
Cao 2011	1	57	0	51	12.9%	2.73 [0.11, 68.63]
Tsimmoyiannis 2010	2	20	1	20	22.6%	2.11 [0.18, 25.35]
Phillips 2011	1	117	1	80	29.6%	0.68 [0.04, 11.05]
Total (95%CI)		438		401	100.0%	2.14 [0.65, 7.06]
Total events	7		2			

Heterogeneity: $\chi^2 = 0.84$, $df = 5$ ($P = 0.97$); $I^2 = 0\%$

Test for overall effect: $Z = 1.26$ ($P = 0.21$)

Odds ratio M-H, Fixed, 95%CI

0.001 0.1 1 10 1000

Favours SPLC Favours CLC

Figure 3 Forest plot. A: Outcome: BDI; B: Outcome: Overall biliary complication. SPLC: Single-port laparoscopic cholecystectomy; BDI: Bile duct injuries; CLC: Conventional laparoscopic cholecystectomy.

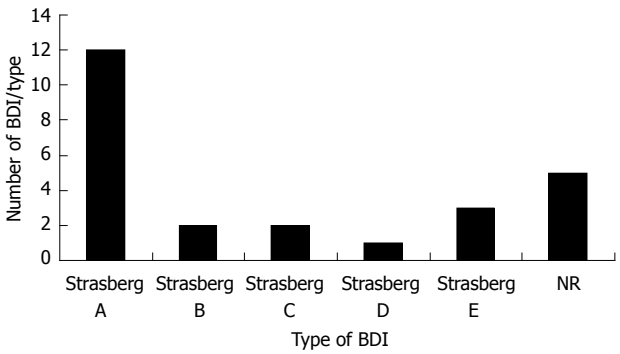

Figure 4 Repartition of the typeof bile duct injuries. BDI: Bile duct injuries.

ongoing allegation that research in surgery is not more than a "comic opera" requires that the surgical community has to make efforts to realize high quality trials. An important shortcoming of published series is the lack of a long-term follow-up, meaning that late occurring bile duct complications may be underreported. Only three studies reported a follow-up of $\geqslant$ 6 mo[10,13,18]. One third of the reports described follow-up of $\leqslant$ 2 wk. Of greatest relevance, even after pooled analysis, is that few patients could be satisfactorily included in the meta-analysis. This is probably the main limitation of our current review. Indeed, since the incidence of BDI is < 1%, large patient numbers are needed to detect the true incidences and its differences. A power calculation based on previous retrospective data[6] revealed a total of 14048 patients would be needed in order to detect a statistically significant difference in terms of BDI rate. This means that all RCT included in this review were clearly underpowered and sample sizes were too small to assess events with a low incidence. As seen with historical comparisons between laparoscopic and open cholecystectomy, RCT will possibly never answer this thorny issue and a larger international prospective database will be more appropriate in this setting.

Table 1 Data of the non-randomized trials

Serie	Year	*n*	BDI	Overall compl
Cuesta *et al*[20]	2008	10	0	0
Palanivelu *et al*[21]	2008	10	1	2
Rao *et al*[22]	2008	20	0	0
Hodgett *et al*[23]	2009	29	0	3
Hong *et al*[24]	2009	15	0	0
Kravetz *et al*[25]	2009	20	0	0
Kuon Lee *et al*[26]	2009	37	1	2
Langwieler *et al*[27]	2009	14	0	0
Merchant *et al*[28]	2009	21	0	0
Philipp *et al*[29]	2009	29	0	6
Podolsky *et al*[30]	2009	15	0	3
Tacchino *et al*[31]	2009	12	0	2
Vidal *et al*[32]	2009	19	0	0
Zhu *et al*[33]	2009	10	0	0
Garijo Alvarez *et al*[34]	2010	30	1	3
Brody *et al*[35]	2010	56	0	2
Carr *et al*[36]	2010	60	0	4
Chow *et al*[37]	2010	41	1	1
Curcillo *et al*[38]	2010	297	1	26
Edwards *et al*[39]	2010	80	3	7
Elsey *et al*[40]	2010	238	0	5
Erbella *et al*[41]	2010	100	0	0
Fronza *et al*[42]	2010	25	0	3
Fumagalli *et al*[43]	2010	21	0	2
Hu *et al*[44]	2010	32	0	0
Ito *et al*[45]	2010	31	0	0
Rawlings *et al*[46]	2010	54	0	2
Rivas *et al*[47]	2010	100	1	1
Roberts *et al*[48]	2010	56	2	3
Romanelli *et al*[49]	2010	22	0	1
Roy *et al*[50]	2010	50	1	2
Schlager *et al*[51]	2010	20	1	1
Kim *et al*[52]	2010	56	1	2
Yu *et al*[53]	2010	33	0	0
Duron *et al*[54]	2011	43	0	0
Han *et al*[55]	2011	150	2	15
Jacob *et al*[56]	2011	36	1	2
Khambaty *et al*[57]	2011	81	0	0
Kilian *et al*[58]	2011	16	0	0
Krajinovic *et al*[59]	2011	50	0	5
Kupcsulik *et al*[60]	2011	30	0	1
Li *et al*[61]	2011	51	0	8
Mesas Burgos *et al*[62]	2011	10	0	0
Mutter *et al*[63]	2011	61	0	0
Prasad *et al*[64]	2011	100	0	0
Qiu *et al*[65]	2011	56	0	3
Raakow *et al*[66]	2011	200	2	11
Rup *et al*[67]	2011	101	0	5
Vermulapalli *et al*[68]	2011	205	3	9
Vrzgula *et al*[69]	2011	100	1	5
Wen *et al*[70]	2011	50	0	2
Wu *et al*[71]	2011	100	1	3
El-geidie *et al*[72]	2012	67	0	1
Feinberg *et al*[73]	2012	50	0	2
Kehagias *et al*[74]	2012	60	0	0
Koo *et al*[75]	2012	100	0	6
Oruc *et al*[76]	2012	25	0	1
Sasaki *et al*[77]	2012	114	1	10
Wong *et al*[78]	2012	20	0	1
Yeo *et al*[79]	2012	60	1	7
Total		3599	26	180
			0.7%	5.0%

BDI: Bile duct injuries.

Unable to assess clearly the safety issue of SPLC based on the meta-analysis, we decided to perform a second enquiry including larger non-randomized prospective and retrospective studies[20-79] in order to increase the sample size, accepting a lower grade of evidence and a higher risk of bias. The incidence of BDI was then found to be as high as 0.7%, three-times greater than the majority of recent large reviews concerning conventional laparoscopy[81-83]. This result was slightly lower than the rate reported by Joseph *et al*[6] in their analysis, probably due to a larger sample size and more recent included series. Ominously, one third (8/25) of the BDI reported in our retrospective review were Strasberg type-B or more, indicating a possible tendency for more complex injuries (Figure 4). This aspect could be underestimated, as 20% of BDI were not described or classified by the authors.

Being aware of a potentially increased rate of BDI, different strategies have been proposed to decrease this unacceptably high risk. Only three out of eleven studies used IOC, with suboptimal technical success rate (62%, 77/124 attempts)[14,16,18]. This may be explained by the need of precision and dexterity required to perform IOC, both lacking in SPLC setting. However, the true impact of this aspect remains unclear, as the discussion on the role of IOC during cholecystectomy is a never-ending story[84,85]. Although described for many years as a critical step to limit BDI during CLC, the use of the so-called "critical view of safety" was clearly reported as *a sine qua non* condition before clipping and dividing the cystic duct in three trials only[11,14,18]. Most often, this was done by the exchange of the dissecting tools for an extra-grasper, in order to gain the right exposure.

Finally, more attention should be paid by the surgeons in critically evaluating their experience with SPLC and in defining the criteria for safety. This aspect is still clearly inadequate in the current literature, as eight studies of the retrospective pool concluded that SPLC was safe, while reporting BDI or increased overall complication rates. Moreover, the hypothesis that the avoidance of 5 mm trocars as in SPLC is of significant clinical benefit was challenged by no-one.

In conclusion, the BDI rate during SPLC seems to be comparable to standard CLC at first sight, but the overall quality of RCT remains low, failing to present any convincing evidence thus far. Larger retrospective data confirm the doubt about the safety of these procedures. Based on the current evidence, SPLC cannot currently be recommended as standard technique for laparoscopic cholecystectomy.

ACKNOWLEDGMENTS

Dr Michael Cotton made a complete revision of our manuscript, looking at language accuracy.

COMMENTS

Background

Single port laparoscopic cholecystectomy (SPLC) is a new technical refinement of endoscopic surgery, which has emerged more than five years ago. The basic

concept of laparoscopy remains intact, but all the instruments are introduced in the abdominal cavity through one single site (usually the umbilicus, but other locations have also been described). The goal of this modification is to decrease the surgical trauma on the abdominal wall, by decreasing the number of necessary ports. On the other hand, due to the loss of triangulation between the instruments, this technique appears technically more difficult to perform.

Research frontiers

Early in its use, this technique was used to perform cholecystectomy. After many years of practice, literature failed to shows important clinical advantages (in terms of pain reduction, length of hospital stay and cosmetic results), when compared to conventional laparoscopic cholecystectomy.

Innovations and breakthroughs

Inadvertent events after SPLC have already largely been covered in other meta-analyses. They concluded that the complication rate was similar between this approach, when compared to conventional laparoscopy. However, these studies were not specifically focused on bile duct injuries. Because this type of complication carries a much more significant potential of decreased quality of life and even mortality, authors considered that safety of SPLC should be evaluated in the light of bile duct injuries (BDI) rate, instead of overall complication rate.

Applications

As long as no stronger evidence concerning biliary safety is provided, SPLC cannot currently be recommended as standard technique for laparoscopic cholecystectomy. It appears mandatory that future studies should focus on longer follow-up of the patients. Owing to the relatively low incidence of complications, it seems evident that only large prospective nationwide cohort studies will be adequate to meet the endpoint of biliary safety. Randomized controlled trials will invariably fail to enroll enough patients to delineate such a small difference.

Peer review

Two peer reviewers contributed to the revision of this manuscript. They recognized that this serious complication should be carefully assessed, before considering a wide acceptation of this new surgical approach. If the conclusion of this manuscript is that literature failed to close the debate, this does not imply a rejection of this new technique. On the contrary, more efforts should be made to continue evaluating SPLC in the light of biliary complications. According to their comments, details on the type of BDI were added. In particular, the distribution of these complications, according to Strasberg classification, was detailed in the results and discussed in the conclusion. Moreover, more information was given concerning the distribution of overall complications.

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CASE REPORT

A case report of anaplastic carcinoma of the pancreas with remarkable intraductal tumor growth into the main pancreatic duct

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Abstract

We herein report a case of anaplastic carcinoma of the pancreas with remarkable intraductal tumor growth into the main pancreatic duct. A 76-year-old male was referred to our hospital for treatment of a pancreatic tumor. Preoperative examinations revealed a poorly defined tumor in the main pancreatic duct in the body of the pancreas, accompanied with severe dilatation of the main pancreatic duct, which was diagnosed as an intraductal papillary-mucinous neoplasm. We performed distal pancreatectomy and splenectomy. The pathological examination revealed that the tumor consisted of a mixture of anaplastic carcinoma (giant cell type) and adenocarcinoma in the pancreas. There was a papillary projecting tumor composed of anaplastic carcinoma in the dilated main pancreatic duct. The patient is now receiving chemotherapy because liver metastasis was detected 12 mo after surgery. In this case, we could observe a remarkable intraductal tumor growth into the main pancreatic duct. We also discuss the pathogenesis and characteristics of this rare tumor with specific tumor growth.

Key words: Anaplastic carcinoma; Giant cell carcinoma; Intraductal tumor growth; Papillary projecting tumor

Core tip: A very rare case of anaplastic carcinoma with remarkable intraductal tumor growth into the main pancreatic duct is reported. This case presented unique features and might help us to better understand the pathogenesis of this rare entity.

Okazaki M, Makino I, Kitagawa H, Nakanuma S, Hayashi H, Nakagawara H, Miyashita T, Tajima H, Takamura H, Ohta T. A case report of anaplastic carcinoma of the pancreas with remarkable intraductal tumor growth into the main pancreatic duct. *World J Gastroenterol* 2014; 20(3): 852-856 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i3/852.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i3.852

INTRODUCTION

Anaplastic carcinoma of the pancreas is an aggressive tumor. The incidence of anaplastic carcinoma varies from 2.1% to 6.8% among reported case series[1-5]. It was first reported by Sommers and Meissner as pleomorphic carcinoma[6]. Pathologically, it is classified into spindle cell, giant cell and pleomorphic types. When detected, patients usually have huge tumors showing rapid growth,

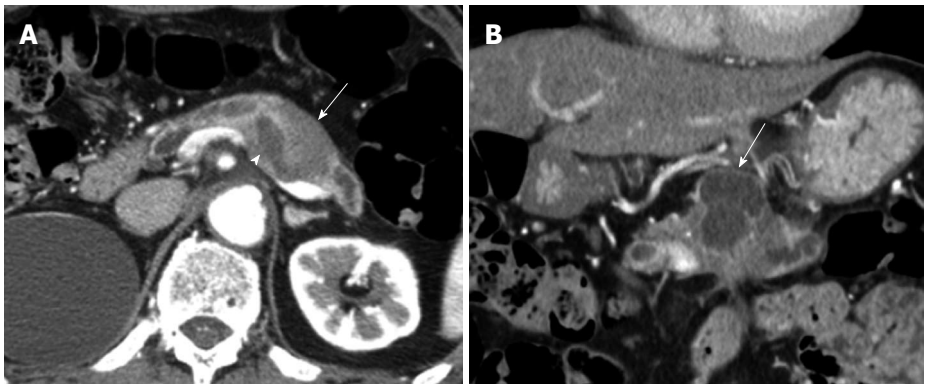

Figure 1 Abdominal computed tomography. A: An axial section image of an abdominal computed tomography showed dilatation of the main pancreatic duct (arrow head) and the adjacent solid tumor (arrow); B: The coronal section image revealed a cystic lesion measuring 6.0 cm × 3.5 cm in the body and tail of the pancreas (arrow).

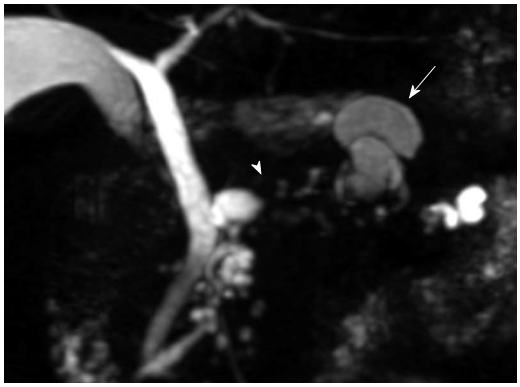

Figure 2 Magnetic resonance cholangiopancreatography revealed a segmental defect of the main pancreatic duct by the solid tumor (arrow head) associated with the multilocular cystic lesion (arrow) in the body and tail of the pancreas.

which are associated with a very poor prognosis. It usually presents as a large cystic pancreatic tumor with areas of hemorrhage and necrosis. En-bloc surgical resection is the only appropriate treatment for the tumors[5,7]. We herein report the clinicopathological features of a case of anaplastic carcinoma of the pancreas with remarkable intraductal tumor growth into the main pancreatic duct.

CASE REPORT

A 76-year-old male was admitted to our hospital for further investigation of a pancreatic tumor. He had received treatment for type 2 diabetes mellitus, essential hypertension and severe arteriosclerosis obliterans. He received ultrasonography as a screening examination, which showed a cystic tumor in the body and tail of the pancreas. He had no significant symptoms. The laboratory findings revealed that the serum carbohydrate antigen (CA19-9) was elevated to 57 U/mL, but the other tumor markers, carcinoembryonic antigen and DUPAN-Ⅱ, were all within the normal ranges. Abdominal CT revealed a tumor measuring 6.0 cm × 3.5 cm in the body and tail of the pancreas. It consisted of a multilocular cystic component and a solid tumor in the lumen of the dilated main pancreatic duct (MPD) (Figure 1). No distant metastasis or lymph node swelling were detected. Endoscopic ultrasonography showed a heterogeneous hypoechoic tumor with a cystic component. On abdominal magnetic resonance imaging, the solid tumor presented with low intensity on T1-weighted images and relatively high intensity on T2-weighted images. MR cholangiopancreatography showed a segmental defect of the MPD by the solid tumor associated with the multilocular cystic lesion in the body and tail of the pancreas (Figure 2). Endoscopic retrograde cholangiopancreatography showed significant dilatation of the MPD from the body to the tail of the pancreas, and an elliptic filling defect in the MPD, suggesting the presence of an intraductal tumor in the lumen of the MPD (Figure 3A). Intraductal ultrasonography demonstrated a solid tumor filling the main pancreatic duct and a cystic lesion in the body of the pancreas (Figure 3B). The diagnosis of the exfoliative cytology was adenocarcinoma. We therefore diagnosed the tumor as an intraductal papillary mucinous neoplasm (IPMN) of the pancreas with obvious dilatation of the MPD. At laparotomy, the tumor had invaded into the transverse mesocolon, but dissemination and distant metastasis were not found. We performed distal pancreatectomy with splenectomy. We decided to make the transecting line of the pancreas by conforming it to the tumor extension using intra-operative ultrasonography. When we transected the pancreas, a reddish tumor protruded from the lumen of the MPD in the resected pancreas (Figure 4A). The main tumor, which replaced the body and tail of the pancreas, was dark reddish-brown and white on the surface, and had infiltrated into the adjacent MPD (Figure 4B). There was a papillary tumor associated with the main tumor in the lumen of the MPD. The MPD was significantly dilated by this protruded tumor. Hematoxylin and eosin staining showed that the part of the tumor presenting a white surface consisted of an adenocarcinoma (tub1, tub2) component, and the part of the tumor presenting the dark reddish-brown surface consisted of anaplastic carcinoma (giant cell type). The projecting tumor in the MPD was composed of anaplastic carcinoma (Figure 5). There was moderate dysplasia with no malignancy (pancreatic intraepithelial neoplasm-2) in the epithelium of dilated main pancreatic duct. The pathological diagnosis

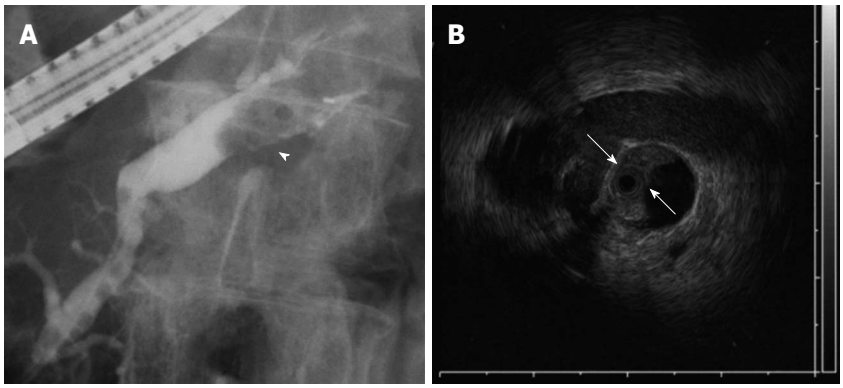

Figure 3 Endoscopic retrograde cholangiopancreatography showed the dilatation of the main pancreatic duct. A: A filling defect in the main pancreatic duct (MPD) (arrow head); B: Intraductal ultrasonography demonstrated a filling lesion in the MPD (arrow).

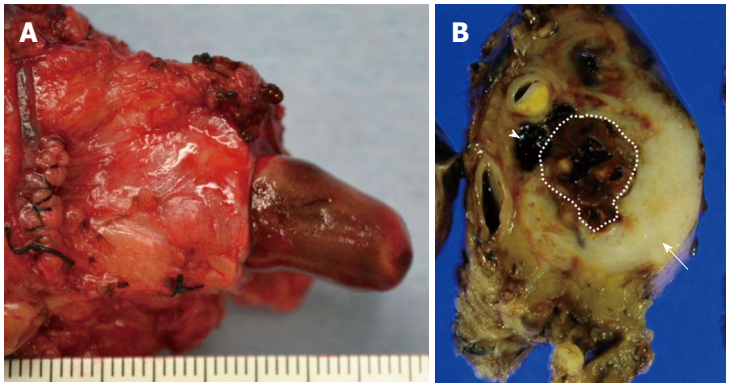

Figure 4 Pathological findings. A: The dilated main pancreatic duct was filled with a papillary projecting tumor; B: The main tumor was dark reddish-brown (arrow head) and white (arrow) on the surface, and showed significant dilatation of the main pancreatic duct (dotted line region).

was a mixture of anaplastic carcinoma (giant cell type) and adenocarcinoma of the pancreas. The postoperative course was uneventful. The patient is now receiving chemotherapy because liver metastasis was detected 12 mo after surgery.

DISCUSSION

Anaplastic carcinoma of the pancreas is a rare and aggressive tumor. Several terms have been used for this tumor, including pleomorphic carcinoma, pleomorphic giant cell carcinoma, pleomorphic large cell carcinoma, sarcomatoid carcinoma and undifferentiated carcinoma[2,3,8]. Three histological variants of anaplastic carcinoma, namely the spindle cell type, pleomorphic cell type and giant cell type, have been described[9]. According to the guideline of the World Health Organization (WHO) in 2010[10], these carcinomas were classified as undifferentiated (anaplastic) carcinomas. On the other hand, anaplastic carcinoma with osteoclast-like giant cells is classified as a subtype of invasive ductal carcinoma.

The clinical features of anaplastic carcinoma have been reported in the literature. The clinical symptoms induced by this tumor are non-specific, such as abdominal pain, fatigue, jaundice, body weight loss, anorexia and back pain[5], which resemble those induced by adenocarcinoma of the pancreas. In the imaging study, anaplastic carcinomas are usually detected as large, moderately hypervascular and exophytic tumors with large areas of necrosis[2].

The preoperative diagnosis of anaplastic carcinoma of the pancreas is difficult. The preoperative diagnosis for the tumor in our case was IPMN, because it appeared to be a multilocular cystic lesion with obvious dilatation of the MPD. The tumor of our case showed a very characteristic appearance because it consisted of a mixture of components of adenocarcinoma and anaplastic carcinoma. The components of adenocarcinoma presented as a solid tumor. A part of the anaplastic carcinoma component developed cystic degeneration, and the other part penetrated and proliferated into the MPD.

Anaplastic carcinoma with intraductal growth into the MPD is so rare that only 9 cases, including our case, have been reported in the English literature[11-18] (Table 1). Only our case was the giant cell type of the anaplastic carcinoma. All cases except for the cases with the osteoclastic giant cell type were associated with a poor prognosis.

The prognosis of anaplastic carcinoma of the pancreas is worse than that of poorly differentiated ductal adenocarcinoma of the pancreas[3,8,19]. Reyes *et al*[20] reported that the median survival time of pleomorphic giant cell carcinoma patients was three months. Strobel *et al*[4] suggested that the median duration of survival was significantly prolonged af-

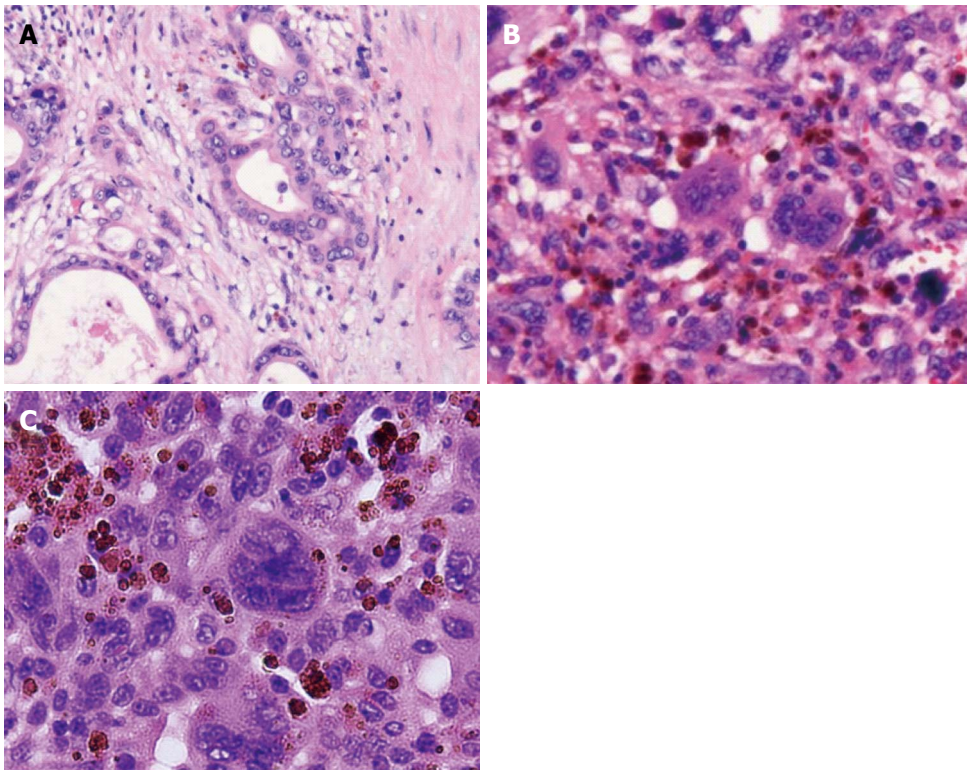

Figure 5 Microscopic appearance of the tumor. Hematoxylin and eosin staining showed adenocarcinoma (tub1, tub2) in the white component, bizarre mono-and multi-nucleated giant cells in the dark reddish-brown component and a projecting tumor into the main pancreatic duct. A: the white component; B: the dark red-brown component; C: the projecting tumor.

Table 1 Reported cases of anaplastic carcinoma with intraductal growth in the main pancreatic duct

Case	Author	Year	Age (yr)	Sex	Location	Size (mm)	Subtype	Treatment	Outcome (mo)
1	Higuchi *et al*[11]	2004	65	F	Pbt	110	spindle	DP	dead (4)
2	Tezuka *et al*[12]	2006	68	F	Ph	42	osteoclastic	PD	alive (22)
3	Suzuki *et al*[13]	2007	71	M	Ph	35	plemorphic	PPPD	dead (1)
4	Kuroda *et al*[14]	2007	59	M	Ph	100	plemorphic	PD	dead (2)
5	Nara *et al*[15]	2009	79	F	Phb	116	osteoclastic	PPPD	alive (14)
6	Maksymov *et al*[16]	2011	68	F	Ph	30	osteoclastic	PD	alive (36)
7	Ishii *et al*[17]	2012	61	M	Ph	32	osteoclastic	PD	alive (14)
8	Yamano *et al*[18]	2013	63	F	Pb	850	plemorphic	DP	dead (4)
9	Our case	2013	76	M	Pbt	600	giant cell	DP	alive (12)

M: Male; F: Female; Ph: Pancreatic head; Pb: Pancreatic body; Pt: Pancreatic tail; Phb: Pancreatic head and body; Pbt: Pancreatic body and tail; DP: Distal pancreatectomy; PD: Pancreatoduodenectomy; PPPD: Pylorus-preserving pancreatoduodenectomy.

ter R0/R1 resection, as compared with palliative surgery (7.1 mo *vs* 2.3 mo). We recommend that patients with anaplastic carcinomas of the pancreas should be offered pancreatic resection whenever possible. Due to its aggressive nature and ability to rapidly recur, the benefits of radiotherapy and chemotherapy have not yet been demonstrated[21,22]. Sporadic case reports have demonstrated a reduction of the tumor mass and prolongation of survival by treatments with 5-fluorouracil, gemcitabine, paclitaxel and radiation[23-25]. However, there is insufficient evidence to recommend any particular treatment, except for surgical resection.

In conclusion, we herein reported a very rare case of anaplastic carcinoma with remarkable intraductal tumor growth into the MPD. This case presented unique features and might help us to better understand the pathogenesis of this rare entity.

COMMENTS

Case characteristics

A 76-year-old male was admitted to our hospital for further investigation of a pancreatic tumor, but he had no significant symptoms.

Clinical diagnosis

This case was diagnosed as an intraductal papillary mucinous neoplasm of the pancreas.

Differential diagnosis

Adenocarcinoma of pancreatic was mentioned in the differential diagnosis.

Laboratory diagnosis

The laboratory findings revealed that the serum carbohydrate antigen was elevated to 57 U/mL, but the other tumor markers, carcinoembryonic antigen and DUPAN-Ⅱ, were all within the normal ranges.

Imaging diagnosis

Computed tomography, endoscopic ultrasonography, magnetic resonance imaging, endoscopic retrograde cholangiopancreatography and intraductal ultrasonography showed significant dilatation of the main pancreatic duct (MPD) from the body to the tail of the pancreas, and an elliptic filling defect in the MPD.

Pathological diagnosis

Hematoxylin and eosin staining showed that the part of the tumor presenting a white surface consisted of an adenocarcinoma (tub1, tub2) component, and the part of the tumor presenting the dark reddish-brown surface consisted of anaplastic carcinoma (giant cell type).

Treatment

Distal pancreatectomy with splenectomy was performed in this case.

Related reports

Only 9 cases of anaplastic carcinoma with intraductal growth into the MPD, including our case, have been reported in the English literature.

Term explanation

Anaplastic carcinoma with intraductal growth into the MPD is rare.

Experiences and lessons

Anaplastic carcinomas are highly malignant tumors, and the authors will follow up the patient closely and continuously.

Peer review

This case presented unique features and might help us to better understand the pathogenesis of this rare entity.

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CASE REPORT

Primary effusion lymphoma-like lymphoma in a patient with inflammatory bowel disease

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Abstract

A 77-year-old man with inflammatory bowel disease (IBD) and who was treated with anti-tumor necrosis factor (TNF), 6-mercaptopurine and corticosteroids, presented with primary effusion lymphoma-like lymphoma (PEL-like lymphoma) with massive ascites. The patient's clinical course was complicated by acute renal insufficiency and hypotension, which led to death within 2 wk. In general, patients with IBD may have an increased risk for development of lymphoma, which is frequently associated with immunosuppressive and/or anti-TNF antibody therapies. PEL is a rare subset of lymphoma localized to serous body cavities, lacks tumor mass or nodal involvement, and is associated with infection by human herpes virus 8 (HHV-8). Primary neoplastic effusion may also be present in patients with large B-cell lymphoma without evidence of human immunodeficiency virus or HHV-8 infections. This type of lymphoma is classified as PEL-like lymphoma. Both PEL and PEL-like lymphoma types have been reported in patients undergoing immunosuppressive therapy, but to the best of our knowledge, the case described herein represents the first PEL-like lymphoma occurring in a patient with IBD.

Key words: Inflammatory bowel disease; Lymphoma; Primary effusion lymphoma; Primary effusion lymphoma-like lymphoma; Immunosuppressive therapy

Core tip: We report a case of primary effusion lymphoma (PEL)-like lymphoma in an elderly, male inflammatory bowel disease (IBD) patient on 6-mercaptopurine (6-MP) treatment. This rare lymphoma subtype is localized to serous body cavities without tumor mass formation or nodal involvement, and has been previously reported in individuals with hepatitis C virus or Epstein-Barr virus infection, in patients who underwent organ transplantation, and elderly patients This novel case in an IBD patient illustrates the importance of considering PEL-like lymphoma in IBD patients treated with 6-MP and anti-tumor necrosis factor antibodies who subsequently develop serous body cavity effusion.

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INTRODUCTION

Primary effusion lymphoma (PEL) and PEL-like lymphoma are rare subsets of B cell lymphoma. The infrequent cases described in the literature have involved immunodeficient individuals [including patients positive for human

immunodeficiency virus (HIV), who underwent transplant, or were undergoing immunosuppressive treatment][1,2], individuals with concomitant infection [including Epstein-Barr virus (EBV)][2-5] or hepatitis C virus (HCV)][2,4-10], cirrhotic[4,6,11] and elderly[4,6,12] individuals. To date however, no case of these rare lymphomas has involved a patient with inflammatory bowel disease (IBD).

Since its initial description as a distinct clinical entity in 1996[13], PEL has been classified as a subtype of lymphoma and characterized as strongly associated with human herpes virus 8 (HHV-8) infection. In contrast, the PEL-like lymphoma subtype, first described in 2008, appears unrelated to HHV-8 infection[1]. Lymphoma is a recognized complication of IBD, most frequently occurring in the form of diffuse large B cell lymphoma. Yet, according to cohort population studies it is debatable whether IBD patients have an increased risk of lymphoma compared with the general population[14-27]. Thiopurine immunosuppressive therapy alone or in combination with anti-tumor necrosis factor (TNF) antibody has rarely been associated with the development of lymphoma in IBD[18-20,26-33]. In young IBD patients, anti-TNF treatment may be associated with rare forms of T cell lymphoma, such as hepatosplenic lymphoma and natural killer T cell lymphoma[34-37].

Herein, we report the occurrence of PEL-like lymphoma in an elderly IBD patient on immunosuppressive therapy. The patient developed massive ascites, fluid cytology of which showed CD20-, BCL2, and vimentin positive large lymphocytes consistent with PEL-like lymphoma.

CASE REPORT

A 77-year-old man with a 3-year history of IBD presented with fever (peak at 39.5 ℃) and dyspnea. The ongoing IBD condition had been originally diagnosed following a sustained bout of severe and frequent bloody diarrhea. The patient's medical history included ischemic heart disease and mitral valve annuloplasty due to mitral valve thickening with myxomatous changes and significant regurgitation. At the initial admission, the patient's serum C-reactive protein (CRP) levels were markedly elevated (78 mg/dL) and the hemoglobin level was slightly below the normal range (13.1 g/dL; normal: 13.8-17.2 g/dL). Colonoscopy showed active left-sided colitis and diverticulosis, and the colonoscopic biopsies showed chronic ulcerative colitis.

An initial treatment of 5-aminosalicylate (4 g/d) had been administered, but the patient showed no response and oral corticosteroid therapy was initiated and provided good results. However, steroid dependency was observed. A tuberculin test was normal and serological tests for hepatitis C antibodies and hepatitis B surface antigen were negative. Anti-TNF therapy (infliximab) had then been initiated but produced only a moderate response and was discontinued after the third dose due to an allergic reaction and only a moderate response.

A second colonoscopy performed 1 year after the initial diagnosis and treatment of IBD showed no signs of active endoscopic colitis, although diverticulosis of the left colon and two small sporadic adenomas of the right colon were observed, together with normal terminal ileum. Histopathological analysis of current biopsies taken from the hepatic flexure to the rectum showed chronic inflammation, crypt abscesses, and multinucleated giant cells. Two years after the initial diagnosis of IBD (and 1 year before the most recent admission), treatment with 6-mercaptopurine (6-MP; 50 mg/d) was administered.

At the patient's most recent clinic presentation, physical examination revealed diminished inspiratory breath sounds on auscultation and increased dullness on percussion of the left lung field. Heart auscultation revealed a mild systolic murmur in the mitral valve area. The patient's abdomen was distended and mildly tender in the left lower quadrant, with slightly increased peristaltic sounds. Shifting dullness consistent with moderate ascites was observed. No palpable organomegaly, masses, or enlarged lymph nodes were detected.

The laboratory test results were as follows: elevated CRP level (68 mg/dL); decreased hemoglobin level (11.6 g/dL); low hematocrit (34%; normal range: 38.8%-50.0%); low white blood cell (WBC) count (2.09×10^3/μL with 1400 neutrophils/μL; normal range: 3.50-10.50 $\times 10^3$/μL); normal platelet count (232000/μL; normal range: 150000-450000/μL); high erythrocyte sedimentation rate (20 mm/h; normal range: 0-17 mm/h); high creatinine level (1.7 mg/dL; normal range: 0.5-1.1 mg/dL); low serum protein level (5.02 g/dL; normal range: 6-8 g/dL); and low albumin level (2.7 g/dL; normal range: 3.4-5.6 g/dL). Serum levels of alkaline phosphatase, glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, and lactate dehydrogenase were all within normal range. Serum was negative for EBV (IgM), HCV, hepatitis B virus (HBV) and HIV antibodies, and positive for cytomegalic virus IgM antibody with high avidity. Serologic tests for *Brucella*, *Mycoplasma*, and varicella-zoster virus were negative. Carbohydrate antigen (CA) 19-9, carcinoembryonic antigen, and alpha-fetoprotein levels were within normal range, whereas the CA125 level was high (516 U/mL; normal limit: < 35 U/mL). An echocardiogram revealed suspicious vegetation on the mitral valve, although this finding was later determined to be incorrect. Urine culture identified *Enterococcus* bacteria and antibiotic therapy (amoxicillin and gentamicin) was administered.

Over the next two weeks, the patient's overall condition deteriorated. Increasing amounts of pleural effusion and massive ascites developed. Chest and abdominal computed tomography showed bilateral pleural effusion, ascites and omental infiltration without enlarged masses or lymph nodes (Figure 1). Doppler ultrasonography of the portal, hepatic and femoral veins showed normal flow without venous thrombosis. Ascites fluid analysis yielded the following results: elevated WBC count (580×10^3/μL; normal limit: < 500×10^3/μL); normal neutrophils count (30×10^3/μL; normal limit: < 250 ×

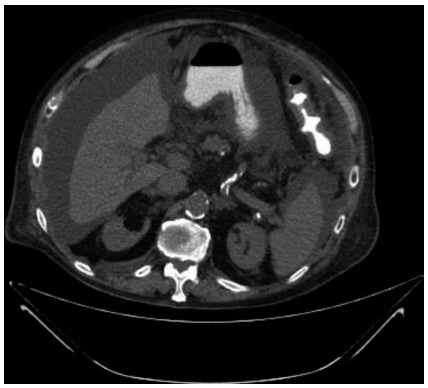

Figure 1 Abdominal computed tomography scan showing marked ascites. No abdominal masses were observed.

$10^3/\mu L$); elevated monocytes count (180 × $10^3/\mu L$; normal limit: < 9% of WBC); elevated atypical lymphocytes count (140 × $10^3/\mu L$; normal value: 0); normal glucose (86 mg/dL; normal limit: > 50 mg/dL); near normal total protein level (2.6 g/dL; normal limit: < 2.5 g/dL); albumin level 1.5 g/dL; high lactate dehydrogenase level (1260 U/L; normal limit: 0.6 of the serum level); normal amylase level (26 U/L; normal limit: < 100 U/L); and normal triglyceride level (16 mg/dL; normal limit: < 200 mg/dL).

Bacterial culturing of ascites fluid provided negative results for all species tested, and polymerase chain reaction for *Mycobacterium tuberculosis* was negative. Cytologic examination of the clear yellow ascites fluid showed enlarged cells with large nuclei, macronucleoli, and abundant cytoplasm (Figure 2A). Immunohistochemical analysis showed negativity for HHV-8 latent nuclear antigen expression. Immunophenotypically, the cells were positive for CD20 (Figure 2B), BCL-2 and vimentin. Flow cytometry revealed CD20- and CD19-positive and CD10-, CD38-, CD56-negative large B cells.

Collectively, these data were consistent with a diagnosis of large B cell lymphoma. After 10 d of admission the patient developed hypotension with acute renal failure, which was attributed to the gentamicin treatment. Despite treatment with intravenous norepinephrine and ascites fluid drainage with intravenous albumin infusion the renal failure became aggravated. The patient underwent hemodialysis but succumbed to the lethal disease course at 14 d after the most recent admission.

DISCUSSION

An increased risk of lymphoma in IBD patients has been reported in several studies[14-20,33,38,39]; in contrast, more recent studies did not show a significantly increased risk of lymphoma in IBD patients compared with the general population[16,17,20-27,38]. Thus, the high risk of lymphoma in IBD patients compared with the general population is still debated. However, the use of thiopurine and anti-TNF alone or in combination is known to be associated with a 2.6- to 5.28-fold increased risk of lymphoma in IBD

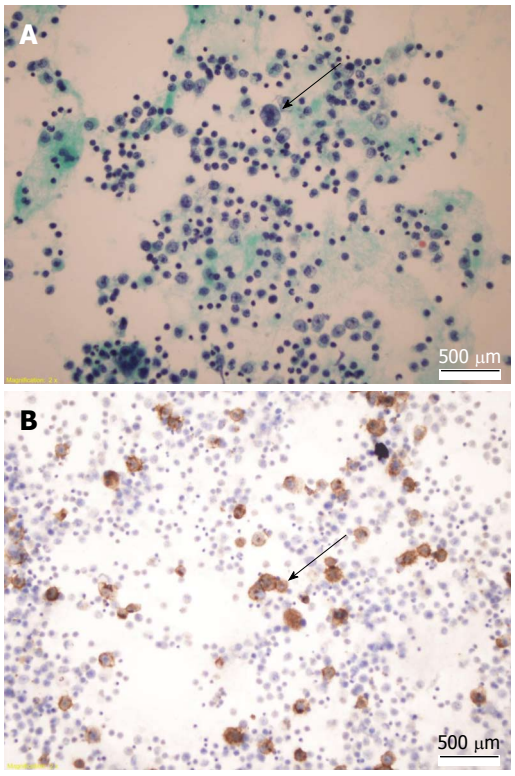

Figure 2 Cytological analysis of the ascitic fluid. A: Papanicolaou staining showed a few large immunoblastic-like atypical cells with large nuclei and prominent nucleoli (arrow). Magnification: × 400; B: Immunohistochemistry staining showed large, CD20-positive lymphoid cells (arrow). Magnification: × 400.

patients[18,19,29,30]. The standardized incidence ratio (relative to the normal population) for lymphoma in IBD patients who were prescribed anti-TNF[32] was shown to be 5.5, and in another study, a 3-fold higher frequency of lymphoma was found amongst IBD patients given anti-TNF[30]. However, even with the increased risk of lymphoma in patients with IBD on thiopurine immunosuppression and anti-TNF therapy, the overall incidence of lymphoma is low[19,29].

Several cases of drug-induced lymphomas in IBD patients are present in the literature, providing precedence for the current case of 6-MP-related PEL-like lymphoma. Indeed, IBD patients over the age of 65 have been characterized as having higher risk of lymphoma due to thiopurine treatment[18,19]. IBD patients under the age of 50 who received thiopurine have shown less frequent rates of lymphoma, and these cases have been suggested to be associated with infectious mononucleosis (EBV)[18,19,26,30]. Anti-TNF therapy in adolescent male IBD patients has also been suggested as associated with development of the rare hepatosplenic T cell lymphoma[34,36,37]; these T cell-derived tumors are EBV-negative in IBD patients and associated with very poor prognosis[19]. In addition, hepatosplenic T cell lymphoma has been reported as a rare complication in IBD patients and attributed to long-term thiopurine exposure[36]. Finally, a single case of infliximab-induced natural killer T cell lymphoma (CD3-, CD56-, CD30- and EBV-positive) in a young IBD pa-

tient was reported recently[35].

PEL is a relatively rare subtype of B cell lymphoma, accounting for approximately 0.3% of non-Hodgkin's lymphoma in HIV-negative individuals and approximately 4% of non-Hodgkin's lymphoma in HIV-positive patients. Generally, PEL develops in HHV-8-positive patients and may be associated with HIV infection[40]. It is characterized by the involvement of serous body cavities and presents as pleural, peritoneal, and pericardial effusion with no tumor mass or nodal involvement[2,11]. Its phenotypic characterization includes negative expression of B cell- and T cell-associated antigens (such as the classic B-cell markers CD20 and BCL-2[1,41,42]) and positive expression of activation markers (such as CD30, CD38 and CD71) and epithelial membrane antigen and plasma cell markers (such as CD138)[2,5]. However, immunophenotyping studies have shown that it is represented by a monoclonal B cell population[42].

HHV-8 and EBV viral infections are considered etiological factors for the development of PEL. Several possible mechanisms may explain how HHV-8 promotes oncogenesis in PEL. First, expression of the viral homologue of cyclin D, a latent gene product of the HHV-8 genome in infected cells, can lead to uncontrolled cell division[3]. Second, the HHV-8 encoded protein latency associated nuclear antigen (LANA)-1 exerts its oncogenic activities by binding to the tumor suppressor protein, p53[43]. Third, LANA-1 is also known to block the transforming growth factor-beta signaling pathway[44]. Finally, another HHV-8 encoded protein, LANA-2, is known to inhibit apoptosis[44].

EBV may also induce B cell proliferation and post-transplantation lymphoproliferative disorder through the EBV-associated protein latent membrane protein-1, which causes cell growth and transformation[45,46]. While EBV-related lymphoma has been described in IBD patients treated with thiopurines[46,47] and the majority of PEL cases in the literature show evidence of EBV infection, the precise role of this virus in PEL oncogenesis remains unclear.

According to the World Health Organization, PEL is only related to HHV-8-positive primary lymphomatous effusion[1]. However, primary neoplastic effusion has also been demonstrated in cases of Burkitt's lymphoma (CD10-positive) and of large B cell lymphoma (HIV-negative and HHV-8-independent)[1]. In the present case, the lymphomatous effusion cells were found to be devoid of HHV-8 infection and displayed morphological and immunophenotypic features of large B cell lymphoma, consistent with the diagnosis of PEL-like lymphoma[1,41].

The majority of PEL-like lymphoma cases reported in the literature mainly involve elderly or immunocompromised patients[1,2,11,40]. Those cases involving immunocompetent patients show a trend of concomitant HCV or EBV infection (19%-42% of patients)[1,2,5,7-11]. HCV infection may cause PEL-like lymphoma by involvement of the CD81 antigen on the cell surface of B cells, which binds to HCV and triggers polyclonal B cell expansion. Subsequent genetic changes may contribute to the development of B cell lymphoma; for example, overexpression of BCL-2 (an anti-apoptotic factor) and HCV-induced translocation can result in deregulation of *PAX5* gene transcription (which encodes the B cell specific antigen)[4,7-10,48]. However, the present case showed no evidence of infection with HCV or EBV.

PEL-like lymphoma accounts for 20% of PEL cases[12]. The definite molecular pathways that underlie the development of malignant lymphomatous effusion in PEL-like lymphoma are still unknown, but are probably similar to those of B cell lymphoma and may include genetic changes such as immunoglobulin and *cMYC* gene rearrangement (translocations), mutations in the *BCL-6* and *P53* genes (pro-apoptotic factor)[2,49-51], and trisomy 8[2,4].

The outcome of HIV-negative, HHV-8-unrelated PEL-like lymphoma is better (median survival: 6-10 mo; 1-year survival rate: 35%) than that of HIV-positive PEL (median survival: 4 mo; 1-year survival rate: 17%)[2]. Treatment for PEL includes combination chemotherapy with cyclophosphamide, doxorubicin, vincristine and prednisone, to which adjunctive treatment with rituximab (anti-CD20 antibody) may be added for PEL-like lymphoma to achieve improved outcomes[3,52,53]. Our patient did not receive chemotherapy because he was critically ill with hypotension and renal failure at the time of diagnosis, which was established only a few days before his death.

In conclusion, we have described the first case of PEL-like lymphoma in an elderly IBD patient on immunosuppressive and biological therapy. We suggest that PEL-like lymphoma can be considered as an additional subset of lymphoma that may rarely complicate the course of IBD treated with immunosuppressive and biologic agents.

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CASE REPORT

Malignant extra-gastrointestinal stromal tumor of the pancreas: Report of two cases and review of the literature

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Abstract

Gastrointestinal stromal tumors (GISTs) are mesenchymal tumors that arise from the gastrointestinal tract. In rare cases, these tumors are found in intra-abdominal sites unrelated to the gastrointestinal tract, such as the mesentery, omentum and retroperitoneum. However, pancreatic extra-gastrointestinal stromal tumors are extremely rare, with only 14 previous cases reported. A 61-year-old man with no clinical symptoms had a routine check-up, during which an abdominal mass located in the pancreas tail was detected. Abdominal surgery was performed with resection of the pancreas tail and the spleen, and he was diagnosed with low-risk GISTs. Another 60-year-old man with no clinical symptoms underwent Computed tomography which revealed a well-demarcated tumor, 6 cm in diameter, in the head of the pancreas. He was diagnosed with pancreatic GISTs. Here, we describe two rare cases of pancreatic GISTs and review the cases previously reported in the literature.

Key words: Gastrointestinal Stromal tumors; Extra-gastrointestinal Stromal Tumors; Pancreatic gastrointestinal stromal tumors

Core tip: Gastrointestinal stromal tumors (GISTs) tend to arise with a higher frequency in the stomach and the small bowel. In fewer than 5% of cases, they originate primarily from EGISTs. Among them, pancreatic GIST is very rare, with only 14 previous cases reported. Here, we report two cases of malignant pancreatic GIST and review the cases previously reported in the literature.

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are mesenchymal neoplasms of gastrointestinal tract, which may occur along the entire length of gastrointestinal tract from the mouth cavity to the anus, and sometimes in the omentum, mesentery, and retroperitoneum[1-4]. They are characterized by high expression of CD-117, a protein encode by c-kit gene. C-kit gene is expressed in 95% of cases; and 60%-70% of tumors are CD34-positive. Pancreatic

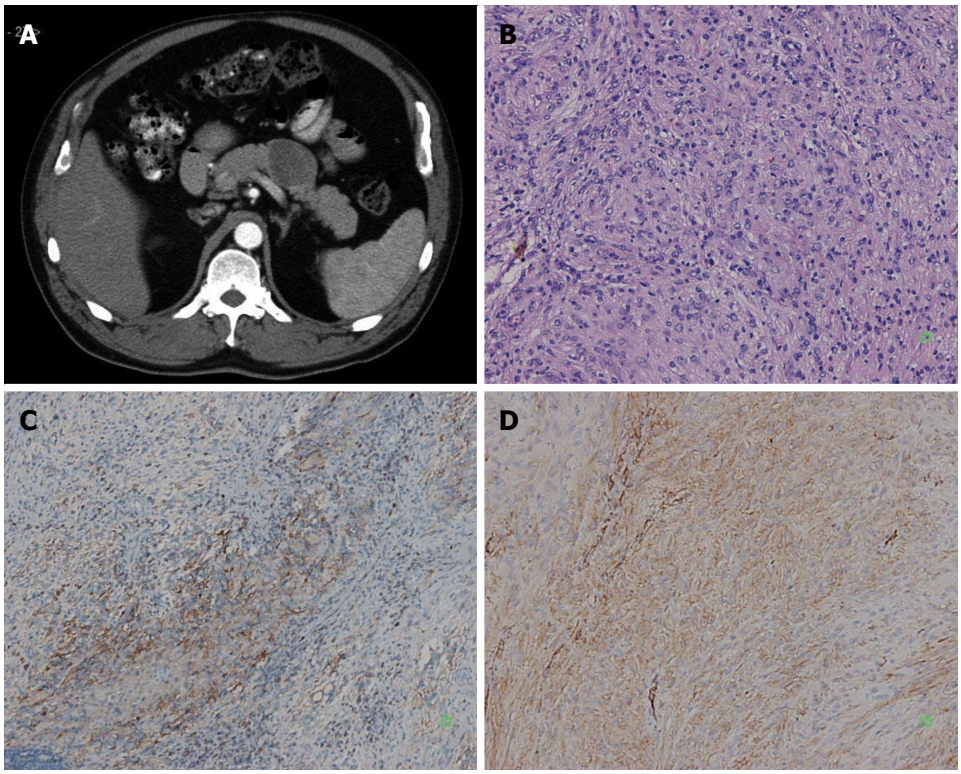

Figure 1 An abdominal mass was detected by ultrasound in a 61-year-old man with no clinical symptoms. A: Enhanced abdominal computed tomography scan showed a solid mass of the pancreatic body, and the tumor located at pancreas tail next to splenic artery; B: The tumor was composed of spindle cell (HE, × 200); C: Immunoreactivity of the tumor cells for CD117 was positive (+) (SP × 100); D: Immunoreactivity of the tumor cells for CD34 was positive (++) (SP × 200).

GISTs are extremely rare, and only 11 cases have been reported in English literature[5-14], two in France literature[15], and only one in Chinese literature[16]. We report two cases of malignant pancreatic GIST and review the cases previously reported in the literature.

CASE REPORT

Case 1

A 61-year-old man with no clinical symptoms had a routine physical examination, during which an abdominal mass was detected by ultrasound. Abdominal computed tomography (CT) revealed a 3 cm × 5 cm mass which is located in the pancreas tail next to the splenic artery (Figure 1A). We did not see any metastasis in the abdominal and pelvic cavity. The tumor was located in pancreatic body-tail，measured 6 cm × 8 cm，and infiltrated the pancreatic tissues. As a result of the unclear nature of the tumor and its close relationship to the splenic artery, a distal pancreatectomy with splenectomy was performed. Inoperative cytology fine needle aspirations found no tumor cells. Microscopically, the tumor was composed of spindle cells (Figure 1B). The mitotic count was minor 5 mitoses/50 high-power fields. Immunohistochemical staining showed immunoreactivity for CD117 (c-Kit) (Figure 1C), CD34 (Figure 1D), and s-100 protein, whereas cells were completely negative for DOG-1, Desmin, NF, synaptophysin, chromogranin A and cytokeratin. The patient was diagnosed with GISTs (low-risk tumor). The patient had no evidence of recurrence or metastasis during the follow-up > 36 mo after operation.

Case 2

A 60-year-old-man with no clinical symptoms underwent CT (Figure 2A), which revealed a well-demarcated tumor of 3 cm × 5 cm × 5 cm, in the head of the pancreas. A classic Whipple pancreaticoduodenectomy was considered. However, the tumor was found in the uncinate process of pancreas during the operation, sized 4 cm × 5 cm × 6 cm, with part of capsule, and no swollen lymph node was found around the tumor. Benign pancreatic islet cell tumor or duodenal leiomyoma was considered during the operation, and cytology fine needle aspiration did not find any tumor cells. As such, local resection of the tumor was performed. On intraoperative examination, the surgical margin was negative. The patient recovered without any complications. Microscopically, the tumor was composed of spindle cells (Figure 2B). The mitotic count was more 5 mitoses/50 high-power fields. Immunohistochemical staining showed immunoreactivity for CD117 (c-Kit) (Figure 2C), and S100 protein, whereas cells were completely negative for smooth muscle actin, synaptophysin, and cytokeratin. Immunohistochemical staining showed that DOG-1 (Figure 2D) was positive. The patient was diagnosed with GISTs (high-risk tumor). Unfortunately, the patient refused to take imatinib because of poor economic status. One year later, multiple nodules about 0.3-2.0 cm were found on the liver surface. The biggest

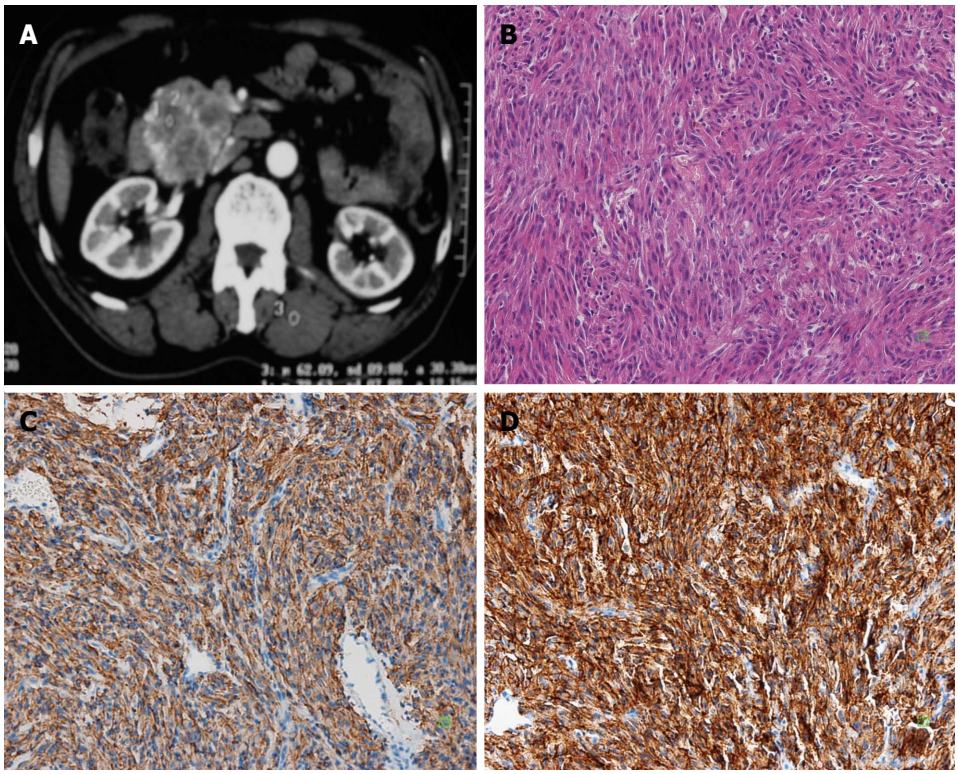

Figure 2 A 60-year-old man with no clinical symptoms underwent computed tomography. A: Enhanced abdominal computed tomography scan showed a solid mass in the pancreatic head; B: The tumor was composed of spindle cell (HE, × 200); C: Immunoreactivity of the tumor cells for CD117 was positive (+) (SP × 200); D: Immunoreactivity of the tumor cells for DOG-1 was positive (+++) (SP × 200).

liver lesion of approximately 2 cm was found in an abdominal CT scan. An excision biopsy showed spindle-shaped tumor cells with atypia, and pancreatic stromal tumor with liver metastasis was considered. The patient was started on imatinib (400 mg *bid*). The last follow-up on radiology three years after the detection of the metastasis, the patient was found with no disease progression.

DISCUSSION

GISTs tend to arise with a higher frequency in the stomach and the small bowel. In fewer than 5% of cases, they originate primarily from extra-gastrointestinal tumors (EGISTs). Among them, pancreatic GIST is very rare, with only 14 previous cases reported (Table 1).

The 14 patients with pancreatic GISTs previously reported aged from 35 to 70 years, averaging 54.2 years, and nine were female. CA19-9 and CEA were mostly normal, which is different from pancreas cancer.

The origin of GISTs currently remains controversial. Most scholars believe that it may originate in Cajal interstitial cells (the gastrointestinal tract pacemaker cells). The expression of tyrosine kinase transmembrane receptor protein c-kit (CD117) is positive in almost all cases of GIST, and this also applies to the pancreas; and 60%-70% of tumors are CD34-positive[8]. Among the 14 cases reported, only one case was CD117 negative in immunohistochemical staining[9], and CD34 was also negative in our cases.

The clinical presentation of pancreatic GISTs varies greatly depending on their size and the presence of mucosal ulceration. The clinical symptoms include abdominal pain, early satiety, flatulence, ileus, bleeding, anemia, and weight loss. Among the previous 14 cases, seven patients experienced epigastric pain, two weaknesses or fatigue, and one nausea and vomiting, whereas four patients had no obvious symptoms, and the tumors were found incidentally. Our two cases had no clinical symptoms, but both underwent CT scanning. Additionally, the patients with tumors located in pancreas head or uncinate process did not have jaundice[5,9,12,13,15], even the tumor size reached nearly 10 cm[9]. Therefore, we conclude that pancreatic GISTs' presentations are concealed, and it is hard to find the tumors in the early stage.

The manifestations of pancreatic GIST are the same as soft tissue tumors. Benign tumors are more definite, their boundaries are clearer and smoother; small tumors typically appear as homogeneous soft tissue masses, while larger tumors often have necrotic centers. Stromal tumors are hypervascular and have no regional lymph node metastasis. These two characteristics are different from those of pancreatic cancer. According to the literature, most pancreatic GISTs cases were diagnosed by surgical biopsy. Endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) is an accurate method of diagnosis. Yan *et al*[13] and Harindhanavudhi *et al*[14] had performed an EUS-FNA biopsy on a pancreatic GIST patient, respectively, which showed that tumor was composed of spindle cells,

Table 1 Previous cases report of Pancreatic gastrointestinal stromal tumor

Ref.	Presentation	Case No.	Location in pancreas	Pathological and immunohistochemical findings	Therapy	Follow-up
Boyer *et al*[15] 2001	Abdominal pain	2	Both of two cases were in the head	A 5.0 cm solid mass with central necrosis; positive for CD117 and CD34; negative for S100	Biopsy of liver lesion and partial duodenopancreatectomy;	NA
Neto *et al*[6] 2004	Epigastric pain, bloating, weight loss	1	Body	A 20.0 cm solid cystic mass with necrotic foci; mitotic count (120/50 HPF); positive for CD117 and CD34; negative for cytokeratins 7 and 20, desmin and synaptophysin	Distal pancreatectomy and splenectomy; treated with imatinib mesylate	Recurrence with peritoneal and retroperitoneal nodal disease 1 mo after surgery
Yamaura *et al*[7] 2004	Incidental finding	1	Tail	A 14.0 cm solid mass with cystic degeneration; few mitoses; positive for CD34 and vimentin; negative for SMA and S100	Distal pancreatectomy splenectomy, and partial gastrectomy	No evidence of disease recurrence at 30 mo
Krska *et al*[8] 2005	Abdominal pain	1	Body and head	A 17.0 cm mass; mitotic count: 1/50 HPF; positive for CD34 and vimentin; negative for S100, chromogranin, actin, and CD117	Partial pancreatectomy	No evidence of disease recurrence at 30 mo
Pauser *et al*[11] 2005	Incidental finding/ abdominal discomfort	2	Tail and body	A 3.0 cm solid mass with positive for CD117 and CD34, cell negative for SMA; A 2.0 cm solid mass with positive for CD117 and CD34, cell negative for SMA	Distal pancreatectomy splenectomy, and partial gastrectomy	No evidence of disease at 24 and 48 mo
Daum *et al*[9] 2005	Incidental finding	1	Head	A 10.0 cm mass with central hemorrhage mitotic count: 2/50 HPF; negative for CD117, vimentin, actin, S100, CD34, desmin, and cytokeratins	Whipple procedure; imatinib	No evidence of disease recurrence at 6 mo
Showalter *et al*[10] 2008	Incidental finding on workup for back pain	1	Tail	A 7.0 cm solid mass; mitotic count: 3/50 HPF; positive for CD117; negative for S100 protein and SMA	Laparoscopic distal pancreatectomy and splenectomy	No evidence of disease recurrence at 27 mo
Yan *et al*[13] 2008	Nausea and vomiting	1	Uncinate process	A 2.4 cm pancreatic mass with spindle cells and mild atypia; positive for CD117; negative for desmin	NA	NA
Harindhanavudhi *et al*[14] 2009	Fatigue and weakness	1	Body	A 16.0 cm × 11.0 cm solid cystic mass; positive for CD117, CD34	Exploratory laparotomy with cystojejunostomy and biopsy of cyst wall	NA
Trabelsi *et al*[12] 2009	Abdominal pain	1	Head	A 10.5 cm × 8.0 cm × 3.0 cm mass with spindle and epithelioid cells Mitotic: 6/50 HPF. Positive for CD117 (c-Kit) and CD34, negative for SMA, S100, synaptophysin and cytokeratins	Hemipancreaticoduodenectomy with antrectomy and partial colectomy	No evidence of disease recurrence at 10 mo
Vij *et al*[5] 2011	Weakness, postprandial	1	Head	A 6.5 cm × 6.0 cm solid mass with pleomorphic Spindle cells; mitosis: 12-15/50 HPF; positive for CD117; negative for CD34, SMA, desmin, S100 and cytokeratins	Whipple procedure; Imatinib on protocol	Developed liver metastasis 24 mo after surgery
Rubin *et al*[16] 2011	fullness and pain abdominal pain	1	Body and tail	A 25.0 cm × 30.0 cm solid mass positive for CD117, CD34 and VIM; negative for S100, NF, DES, SMA, and CK	Distal pancreatectomy splenectomy	NA
Present case 1	Incidental finding	1	Tail	A 6.0 cm × 8.0 cm solid mass with spindle cells mitotic: < 5/50 HPF; positive for CD117, CD34, and s-100 protein, negative for DOG-1, Desmin, NF, synaptophysin, Chromogranin A and cytokeratin	Distal pancreatectomy splenectomy	No evidence of disease recurrence at 36 mo
Present case 2	Incidental finding	1	Head	A 4.0 cm × 5.0 cm × 6.0 cm solid mass with spindle cells mitosis: > 5/50 HPF. Positive for CD117, S100 protein and DOG-1, negative for SMA, synaptophysin, and cytokeratin	Pancreatic head tumor resection	Developed liver metastasis 12 mo after surgery progression-free survival for 36 mo after two times of TACE and oral imatinib

and immunohistochemistry showed that it was strongly positive for CD117, resulting in a preoperative diagnosis of pancreatic stromal tumor. The result was consistent with the postoperative pathological diagnosis.

In terms of the pathology of pancreatic stromal tumors, most undergo expansive growth, resulting in clearly isolated round or oval lumps. Tumors have a diameter of 2-30 cm, averaging about 10 cm according to the previous literature and are encapsulated with a smooth surface or adhesion with the surrounding tissues that are rich in blood vessels. The tumor section was flat, gray or gray-red because of collagen in blood vessels, and hemorrhage. Other changes after dissolution, such as granular surface or presence of small indentation, are different from the smooth muscle tumors; it does not show outward protrusion or swirling, but is soft and delicate, some are even fish-like. GISTs may have hemorrhage, necrosis, cystic degeneration, and other secondary changes, especially when the tumor is large. But it does not lead to malignancy.

Pancreatic GISTs are mainly composed of spindle cells. Malignant GISTs cells have different degrees of atypia, and mitotic figures are visible. Pancreatic GISTs are different from typical smooth muscle and nerve sheath tumors. Immune markers are expressed at low levels, such as smooth muscle actin (30%-40%), desmin (< 5%), neuron specific enolase and S-100 (< 5%). C-Kit gene is expressed in 95% of cases, making it a characteristic of GISTs. And 60%-70% of tumors are CD34-positive[16].

Pancreatic GISTs can be divided into very low, low, intermediate and high risk of metastases according to the tumor size and mitotic counts on histology and immunohistochemistry. Tumors larger than 5 cm with more than 5 mitoses per 50 HPF and tumors larger than 10 cm, regardless of mitotic count, should be considered as lesions with high risk of malignancy[17]. It was assumed that most tumors reported were of high risk (9/14), which may be related to the symptom concealing and expansive growth. The biggest pancreatic GIST reported was nearly 30 cm[15]. However, the patient only presented with a symptom of abdominal uncomfortableness. Moreover, pancreatic GISTs easily develop liver metastasis. In our second case, multiple metastatic nodules were found one year after surgery.

Surgery remains the preferred treatment for pancreatic stromal tumors[9]. Pancreaticoduodenectomy is feasible when there are stromal tumors of the pancreatic head[15]. If the tumor is small with clear boundaries or the patient cannot tolerate pancreaticoduodenectomy, duodenum-preserving pancreatic head resection or simple tumor excision can also be applied. GISTs and EGISTs regional lymph node metastases are rare; thus, regional lymph node dissection is not required generally in surgery[15].

Imatinib (Gleevec), which is an inhibitor of the tyrosine kinase activity of C-Kit, has revolutionized the treatment of this disease and the overall median survival now reaches 5 years[18]. For large GISTs, Gleevec before surgery can reduce tumor burden, increase the rate of complete resection of the tumor, and help improve prognosis[19]. For metastatic or unresectable GISTs, imatinib treatment can significantly reduce the tumor size as well as improve survival rate[20]. Three cases of pancreatic GISTs had been reported taking imatinib after liver metastasis was found, and all these patients had at least a 2-year progression-free survival. Our second case took imatinib (400 mg/d) for only two months because of poor economic status, while the patient also had a progression-free survival for 3 years.

Many factors can affect the prognosis of pancreatic stromal tumors, such as age, location, cell differentiation, C-Kit gene mutations, DNA factors, histological grade, and p53. The prognosis of pancreatic stromal tumors is closely related to the biological behavior of tumors. Four of the 14 studies did not give the information of follow-up[13-16]. Eight cases were found no evidence of recurrence during follow-up (the minimum period of follow-up was six months)[7-12]. Neto *et al*[6] reported a case of peritoneal and retroperitoneal nodal disease with recurrence one month after surgery. Vij *et al*[5] reported one case with liver metastasis 24 mo after surgery. In the present study, in the second case, liver metastases occurred after 1 year; but intervention and treatment with imatinib led to a good survival. The median survival of patients with liver metastasis from pancreatic cancer is only about 6 mo. It is obvious that the prognosis of this tumor is significantly better than that of pancreatic cancer.

In short, pancreatic stromal tumor is rare. The lack of specific clinical manifestations, hypervascular tumor, and regional lymph node metastases, is slightly different from the pancreatic tumor in the imaging characteristics of the two cases. Surgical resection resulted in a significantly better prognosis than that of pancreatic cancer.

COMMENTS

Case characteristics

The two cases presented no clinical symptoms, but both of them underwent computed tomography (CT).

Clinical diagnosis

Both of the two cases were diagnosed with pancreatic tumor.

Differential diagnosis

The two pancreatic Gastrointestinal stromal tumors (GISTs) were diagnosed using immunohistochemical staining.

Imaging diagnosis

Case 1: Abdominal CT revealed a 3 cm × 5 cm mass which is located in the pancreas tail next to the splenic artery; Case 2: Abdominal CT revealed a well-demarcated tumor, 3 cm × 5 cm × 5 cm, in the head of the pancreas.

Pathological diagnosis

Case 1: The patient was diagnosed with a low-risk GIST; Case 2: The patient was diagnosed with a high-risk GIST.

Treatment

Case 1: underwent distal pancreatectomy with splenectomy; Case 2: underwent a tumor local resection; the patient was started on imatinib (400 mg bid) when multiple liver metastases were found one year after surgery.

Term explanation

GISTs are mesenchymal tumors that arise from the gastrointestinal tract.

Experiences and lessons

Pancreatic GIST is very rare, with only 14 previous cases reported. Here, we

report two cases of malignant pancreatic GIST and review the cases previously reported in the literature.

Peer review

This case report is very interesting. In rare GIST cases, these tumors are found in intra-abdominal sites unrelated to the gastrointestinal tract, such as the mesentery, omentum and retroperitoneum, but pancreatic extra-GIST are extremely rare. In this manuscript, Tian *et al* reported two cases of pancreatic GISTs. The cases are well presented, and the discussion is good. It can be accepted for publication after some minor language correction.

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Acknowledgments

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Patent (list all authors)

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

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