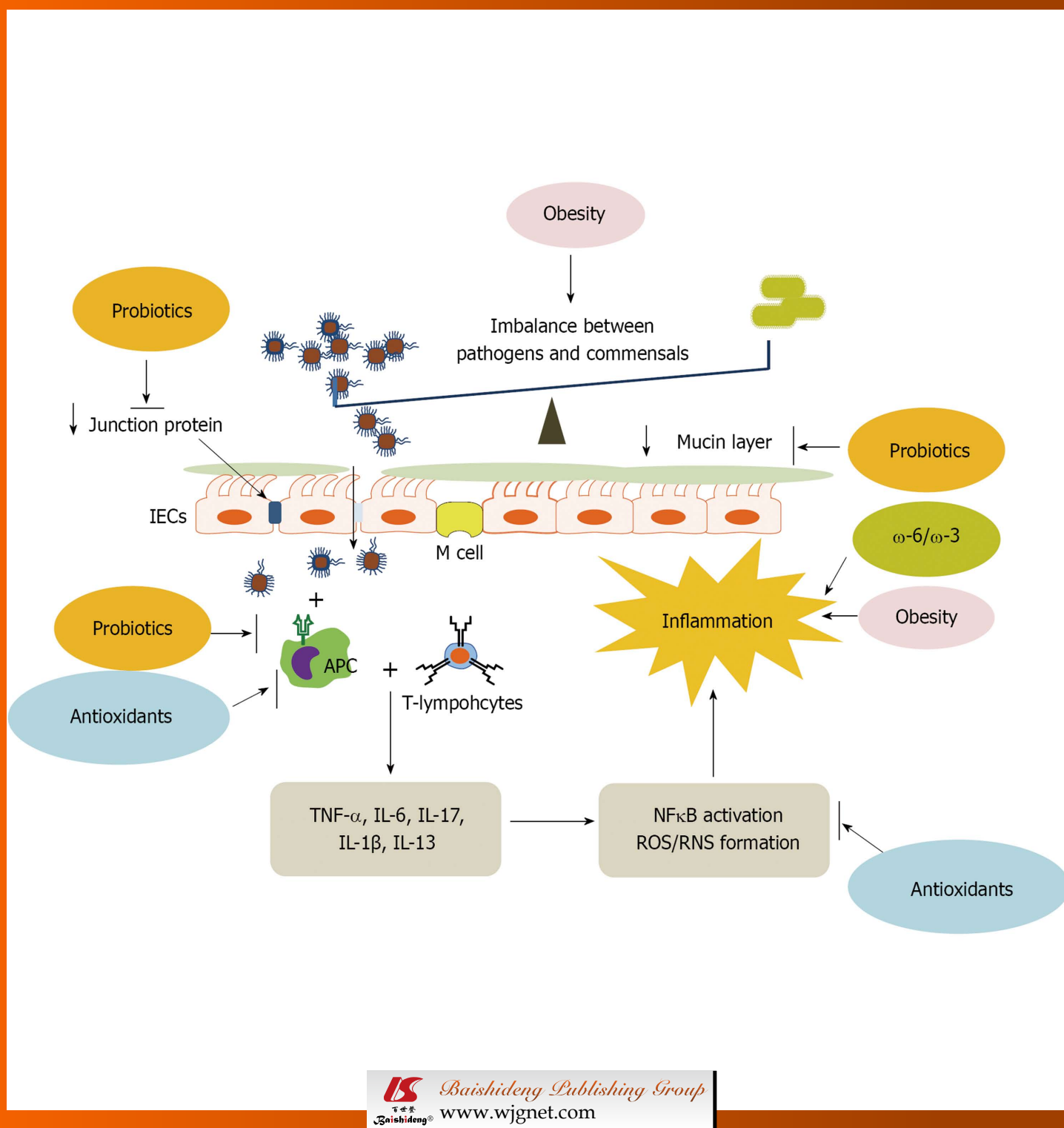


# World Journal of *Gastroenterology*

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## Histological healing in inflammatory bowel disease: A still unfulfilled promise

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incomplete, and somewhat conflicting data exist on this topic, especially because there is still the need to standardize both histological assessment and the severity grading of these disorders; Issues that have not been yet been resolved for clinical practice and therapeutic trials. Hopefully, with the help of an increased awareness on the clinical researchers' side, and the availability of dedicated pathologists on the other side, this matter will be effectively faced and resolved in the near future.

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**Key words:** Crohn's disease; Healing; Histology; Inflammatory bowel diseases; Ulcerative colitis

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### Abstract

Treatment of inflammatory bowel disease (IBD) is traditionally based on several drugs, including salicylates, corticosteroids, and antibiotics; in addition, the therapeutic armamentarium has considerably evolved with the advent of newer, effective therapeutic measures (such as the biological agents) that are able to improve in a considerable manner both the clinical and endoscopic variables. Thus, mucosal healing, at least considered from an endoscopic point of view, is today regarded as the ultimate endpoint for treatment of these conditions. However, it is also increasingly clear that endoscopic healing is not necessarily paralleled by histological healing; There are few doubts that the latter should be considered as a true, objective healing and the ultimate goal to reach when treating patients with IBD. Unfortunately, and surprisingly, only a few,

### INTRODUCTION

The medical treatment of inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), is based on several different approaches relying on aminosalicylates, antibiotics, corticosteroids, and immunomodulatory agents, in addition to the newest biological agents<sup>[1]</sup>. At present, there is also a strong impulse toward treatment paradigms directed to achieve specific targets, such as a decrease in hospital admissions, the need for surgery, and mucosal healing<sup>[2]</sup>. The latter point seems to be particularly important, because reaching this goal would also produce a domino effect, resulting in less complications, hospitalization, and surgical procedures related to IBD<sup>[3]</sup>.

However, mucosal healing could be considered as an ambiguous term, because it may be related to both the endoscopic and histological aspects, and at present

a standardized definition for IBD patients does not exist<sup>[4]</sup>. Actually, by considering mucosal healing in IBD, histological remission is not recommended as primary endpoint for therapeutic trials<sup>[5]</sup>, whereas the suggested endpoint is to obtain a genuine endoscopic healing (defined as absence of friability, blood, erosions, and ulcers in all visualized segments)<sup>[5]</sup>.

Strictly speaking, the optimal treatment goal should be the complete resolution of the inflammatory process, and this is reached only when confirmed by the corresponding histological assessment. In fact, the presence of endoscopic healing does not necessarily imply that this also happens at a microscopic level<sup>[6]</sup>, as also shown (for instance) in celiac disease, in which a gluten-free diet restores the normal endoscopic aspect, but rarely does the microscopic assessment show completely normal features<sup>[7]</sup>.

Moreover, there are objective reasons that probably hamper the use of histological healing as an end point for therapeutic trials. For instance, it has been recently stated that, concerning histopathological assessment of IBD, "...bewildering variations can be observed in the terminology employed to report either individual lesions or diagnostic categories"<sup>[8]</sup>. In addition, significant differences may exist between UC and CD concerning this aspect. In fact, in UC (in which lesions are usually limited to the mucosa) mucosal/histological healing may represent the ultimate therapeutic goal, whereas in CD (a transmural process) mucosal/histological healing should be achieved as a minimum therapeutic target<sup>[9]</sup>. Thus, even considering the potentiality of the new therapeutic approaches, the importance of achieving also histological healing might add further value to future trials<sup>[10]</sup>.

A generally accepted definition of histological mucosal healing does however not exist. Histological healing could be defined as "a normal mucosa" or as a disappearance of inflammation, and hence, "a mucosa with limited architectural abnormalities but normally differentiated epithelial cells and no signs of active inflammation (presence of neutrophils) or an increased density of lymphocytes and plasma cells". Usually, histological assessment, when performed during drug trials, has focused mainly on improvement and regression of inflammatory features. The assessment is usually based on the analysis of microscopic sections stained routinely with haematoxylin and eosin. This is a cheap and simple technique which is widely available.

## ONLINE SEARCH

We made a comprehensive online search of Medline and the Science Citation Index using the keywords "inflammatory bowel disease", "ulcerative colitis", "Crohn's disease", "mesalazine", "immunosuppressant", "biologics", "infliximab", "azathioprine", "corticosteroids", "prednisolone", "beclomethasone", "endoscopy", "histologic", "mucosal", and "healing" in various combinations with the Boolean operators "and", "or", and "not".

We included only articles that related to human studies, and we performed manual cross referencing, selecting articles published in English between January 1965 and April 2012. A search of non-English language articles and journals older than 1965 was also performed in our library. We excluded letters, and we reviewed abstracts only when the full papers were unavailable.

## HISTOPATHOLOGICAL CONSIDERATIONS

An important point is that most literature on this topic originates from only rectal sampling<sup>[6]</sup>. Thus, it is important that the pathologist receives an adequate number of biopsies correctly oriented from several sites, including the rectum and the terminal ileum. The biopsies should always be accompanied by a report including the age of the patient, clinical information, duration of disease, and type of treatment<sup>[11]</sup>.

The pathological assessment of IBD basically relies on two types of lesions, combined with each other in various ways, represented by architectural abnormalities (that include crypt branching/shortening, decreased crypt density, and irregular mucosal surface) and inflammatory features (transmucosal increase of lamina propria mononuclear cells and the presence of epithelioid granulomas)<sup>[6,12]</sup>.

Moreover, it should be always kept in mind that the histological features of IBD are variable in time, due to both the natural evolution of the disease and the different therapeutic measures. For instance, crypt distortion in UC is usually absent in early stages<sup>[13]</sup>, and the development of architectural abnormalities may take up to 2 mo to appear<sup>[14]</sup>. Also, decreased crypt density is usually not observed in the first week of the disease, and it is present in about 75% of UC patients with longstanding disease in remission<sup>[15]</sup>. Again, irregular mucosal surface (present in about 40% of IBD patients) is generally observed in subjects presenting with symptoms for > 2 wk, and it is more common in UC than in CD<sup>[6,16,17]</sup>. Thus, the absence of mucosal architectural abnormalities in the early phases may present difficulties in the differential diagnosis with acute colitis and during follow-up<sup>[18]</sup>.

Concerning inflammatory features, an increase in cellularity of the lamina propria (especially when localized in its basal third) allows us to distinguish longstanding IBD patients from normal subjects in almost 90% of cases<sup>[19]</sup>. Another useful clue is an increase of plasma cells, a population increased in IBD rectal samples compared to controls<sup>[20]</sup>; indeed, blind evaluation of IBD and control specimens has revealed that > 50% of patients displayed basal plasmacytosis<sup>[17]</sup>. However, it must always be kept in mind that inflammatory features also are not constant over time; for instance, a prospective study has demonstrated that focal basal plasmacytosis was found in 40% of IBD patients with symptoms for < 2 wk, but disappeared after 1 year follow-up in half of those without relapse<sup>[13]</sup>. Granulomas, a distinctive feature of CD, are not constantly present, and are more frequent in



**Table 1** Score for histological assessment of ulcerative colitis

No significant inflammation	Mucosa free from active inflammation; no erosions or crypt abscesses; surface and glandular epithelial cells intact; general architecture of the mucosa often disturbed; edema and fibrosis of the lamina propria with occasional foci of lymphocytes
Mild to moderate inflammation	Epithelium usually intact; Glandular tubules irregularly arranged and often showing increased proliferative activity; Edema, vascular congestion, and interstitial hemorrhage presented in the lamina propria; Lymphocytes, plasma cells, and eosinophils increased in number, neutrophils often present, but less numerous than in the more severely affected specimens; Variation in intensity of inflammatory change in individual specimens giving a range of appearances from relatively quiescent to active inflammation
Severe inflammation	Mucosal surface often irregular due to edema, interstitial, hemorrhage, or inflammatory exudate in the lamina, propria; Small epithelial breaches common, sometimes with frank erosions and purulent exudate; Neutrophils and eosinophils passing through the damaged epithelium; Areas of flattened and cuboidal cells especially found near erosions; Mucosa showing heavy interstitial infiltration by lymphocytes, plasma cells, eosinophils and neutrophils; Glandular abnormalities (neutrophilic invasion of the tubules, epithelial focal degeneration and shedding of necrotic/viable cells into the glandular lumina; crypt abscesses (neutrophils, eosinophils, and epithelial debris); Sometimes, breaking down of the wall of the tubule with inflammatory exudate passing from the tubule into the lamina propria

Adapted from Truelove *et al.*<sup>[23]</sup>, 1956.

children and in the early phases of the disease<sup>[6,21]</sup>.

It must also be stressed that in CD, being a transmural disease, mucosal healing may not be sufficient even though the mucosa may return to almost normal after treatment. Deeply situated lesions may persist, and endoscopy with biopsy can however not assess the deeper layers of the digestive tract<sup>[22]</sup>.

An important point to be addressed is that, to date, there is no standardized histological scoring system for the assessment of disease activity in IBD, although many scores have been proposed. A scoring system was firstly introduced many years ago for UC<sup>[23]</sup>, and in subsequent years many other histological scores have been proposed, all based on routine haematoxylin-eosin (HE) staining. As a result of the more homogeneous distribution of the lesions, most of these scores have been described in UC, many during the 1980s<sup>[24-28]</sup>, a few in the 1990s<sup>[29-32]</sup>, and only two more recently<sup>[33,34]</sup>, although some proposal for CD also exist<sup>[35-37]</sup>. However, even recent consensus conferences on CD and UC have not included a formal histological score for the pathological evaluation of these patients<sup>[38-41]</sup>.

This is partly due to the possible impact of sampling error, to the limits of routine HE staining, and to the existence of some poorly known variables. Histologically, disease activity is usually based upon the combination of the presence of neutrophils and epithelial damage, because neutrophils can be recognized reliably and it is known that they release molecules that are capable of damaging the tissue. Other cells, however, might also have deleterious effects. The activity status of cells such as macrophages and lymphocytes can however not be assessed on HE-stained sections. The influence of eosinophils is still unclear. Elevated levels of eosinophils have been observed in colonic biopsy samples from UC patients, and increased numbers of these cells and eosinophil-derived granular proteins (major basic protein, eosinophil cationic protein, eosinophil peroxidase, and eosinophil derived neurotoxin) have been shown to correlate with morphological changes in the gastrointestinal tract, disease severity, and gastrointestinal dysfunction<sup>[42,43]</sup>.

Finally, the natural evolution of the disease and its

histological features are not well known. Inflammatory features may however decrease over time and the distribution pattern may change<sup>[44]</sup>.

## HISTOLOGICAL HEALING IN UC

### Corticosteroids

These agents were introduced early in the therapeutic armamentarium of IBD, and are still commonly used in the acute phases; moreover, their ability to induce at least macroscopic mucosal healing at endoscopy is well recognized<sup>[45]</sup>. Concerning histological healing, only a few studies are available in literature, and none recent.

The first study to tackle this topic in 40 patients with active UC investigated the effect of 1 wk treatment with rectally administered hydrocortisone hemisuccinate sodium<sup>[46]</sup>. Histological variables were analyzed by a previously described scoring system<sup>[23]</sup> (Table 1), and showed significant improvement compared to baseline and to placebo in patients treated with steroids, in whom there was a 50% decrease in severe grading and 64% in moderate grading. Moreover, 55% shifted toward mild grading after treatment. However, no normalization of histological variables was reported in this study. Interestingly, the authors of the histological scoring system stated that "...this system of grading depends to a large extent on subjective judgments"<sup>[23]</sup>.

In a study of 215 patients with UC, cell counts revealed that acute inflammation as indicated by neutrophils was decreased most notably following treatment with prednisone (and/or 6-mercaptopurine). Chronic inflammation as indicated by the presence of plasma cells was also reduced after prednisone and equally after 6-mercaptopurine and salicylazosulfapyridine. The authors further demonstrated an increase of epithelial goblet cells and lamina propria macrophages during healing<sup>[47]</sup>. Although these results are particularly interesting, the method used is time consuming and probably difficult to apply routinely.

Another 2-wk study compared the effects of aqueous hydrocortisone enema and a suspension of hydrocortisone in an inert foam base in 30 UC patients with proctosigmoiditis<sup>[48]</sup>. Histological assessment was carried

**Table 2** Modified scoring system for histological assessment of ulcerative colitis

Inflammation grade	Score	Intensity	Histological criteria
Active inflammation	0	Normal	Neutrophils not present in crypt or surface epithelium and no exudate, erosion or ulceration
	1	Low grade	Neutrophils present transmigrating through the crypt epithelium or within crypt lumina in < 20% of crypts; no erosions or ulcers
	2	Moderate	Neutrophilic infiltration in > 20% of crypts or presence of erosions
	3	High grade	Presence of ulcers
Chronic inflammation	0	No increase	Normal number of chronic inflammatory cells present primarily in the superficial lamina propria
	1	Moderate	Moderate number of mononuclear cells Aggregated between crypts at the base of the lamina propria
Crypt distortion	2	Severe	Marked increase in chronic inflammation shown by sheets of chronic cells
	0	None	Crypts had normal outlines with only artifactual irregularities
	1	Mild	Scattered or rare crypts showing irregular (bent, forked) outline
	2	Moderate	Approximately 25%-50% of crypts with an irregular outline
	3	Severe	> 50% of crypts with an irregular outline

Adapted from Hanauer *et al*<sup>[50]</sup>, 1998.

out by means of an active inflammation score (0-3 for each of the following three characteristics: polymorph infiltration, mucus depletion, and superficial epithelial degeneration, allocating a score of 0-9 to each biopsy). In the enema group there was a significant ( $P < 0.05$ ) improvement in active inflammation score (from 4.4 to 2.5) compared to baseline, but this was not observed in the foam group (inflammation score 5.2 *vs* 4.9). However, this apparent superiority for enema treatment was not confirmed when the two treatments were directly compared.

Lee *et al*<sup>[49]</sup> subsequently compared the effect of mesalazine foam enema (2 g) and prednisolone foam enema (20 mg) in 295 patients with relapsing distal UC. After 4 wk treatment, histological remission (score 0), using the above scoring system<sup>[48]</sup> was obtained in 27% of patients in the mesalazine group and 21% of patients in the prednisolone group.

In another study, 233 patients with active distal UC/proctitis were randomized to either a placebo enema or budesonide enema at a dose of 0.5 mg/100 mL, 2.0 mg/100 mL, or 8.0 mg/100 mL<sup>[50]</sup>. Biopsy specimens were histologically graded for active inflammation, chronic inflammation, and crypt distortion, and graded according to a modification of the Truelove and Richards scoring system<sup>[23]</sup> (Table 2). After treatment, total histopathological score (defined as the sum of the three above components) significantly improved in the 2.0 and 8.0 mg groups, compared to placebo, whereas no differences were found for the 0.5 mg dose. However, remission rate in this study did not include histological remission.

A study conducted on 17 patients with severe UC compared the effect on unfractionated heparin and corticosteroids, and demonstrated an improvement of histopathological grading (that, however, was not formally described), not significantly different between the two treatments (63% in the heparin group and 50% in those treated with steroids) at the end of the study<sup>[51]</sup>.

Rizzello *et al*<sup>[52]</sup> have evaluated the effect of oral 5-aminosalicylic acid (5-ASA) (3.2 g/d) compared to oral

beclometasone dipropionate (5 mg/d) and to placebo in 118 patients with extensive or left-sided mild to moderate UC. After 4 wk of treatment, the histological score<sup>[23]</sup> was significantly improved compared to baseline, but no differences between treatment groups were observed and no specific histological description was given.

In a more recent study, two budesonide formulations (foam and enema) were compared in 449 patients with distal UC/proctitis<sup>[53]</sup>. Histological assessment was conducted according to the score proposed by Riley *et al*<sup>[29]</sup>, which takes into account six features [acute inflammatory cell infiltrate (neutrophils in the lamina propria), crypt abscesses, mucin depletion, surface epithelial integrity, chronic inflammatory cell infiltrate (round cells in the lamina propria), and crypt architectural irregularities], each graded on a 4-point scale (0 = none; 1 = mild; 2 = moderate; 3 = severe). At the end of treatment, a similar histological improvement was obtained in both groups (51% foam, 57% enema). Again, no specific histological description was given.

Overall, by considering the above studies, although steroid treatment seems to improve histological abnormalities in UC patients, true remission rates are probably quite low, and no more than 30%.

### Salicylates

Overall, not many studies are available in the literature that have assessed the effect of these drugs on histological healing in UC, and it should be stressed that these studies included different subtypes of patients and used different pharmacological formulations and modes of administration. The study by Sommers *et al*<sup>[47]</sup> mentioned earlier did show some beneficial effects on inflammatory cell counts in the lamina propria.

The first study to assess this topic was that of Rao *et al*<sup>[54]</sup>, who compared the effects of olsalazine (2 g/d) and sulfasalazine (3 g/d) in 37 patients with mild to moderate active distal UC. The degree of inflammation in rectal biopsies was graded as absent (without inflammation), mild (chronic glandular damage with definite increase in inflammatory cells), moderate (mild inflam-

mation plus small foci of ulceration), or severe (moderate inflammation in the presence of crypt abscesses and widespread ulceration). In both groups, a similar histological improvement (44% for olsalazine and 46% for sulfasalazine) was seen after 1 mo, even though this was not as impressive as the improvement of endoscopic appearance or clinical response. However, no histological details were given in this study, apart from generic statements (improved, unchanged, worsened), nor it was stated whether histological remission was achieved.

In a 12-wk multicenter double-blind parallel group study, the effects of balsalazide (6.75 g/d) or sulfasalazine (3 g/d) were compared in 57 UC patients, stratified for disease severity; topical and/or oral steroids were administered if clinically needed<sup>[55]</sup>. Histological assessment (only graded as 0, normal; 1, mild UC; 2, moderate UC; 3, severe UC) showed a similar improvement in both groups at the end of treatment. No histological details were given.

The same authors carried out another study with the same drug regimens (but without concomitant use of steroids) in 50 patients with mild to moderate UC<sup>[56]</sup>. Pathological assessment was used to identify patients in whom clinical remission was also associated with histological remission. Rectal biopsies, obtained at baseline and after 8 wk) were graded on a 4-point scale (0, normal; 1, minimal inflammation but not active disease; 2, moderate inflammation; 3, severe inflammation); all patients included in the study had at least mild inflammation at baseline, with 58% displaying severe inflammation. After treatment, histological grades improvement was observed in both groups, but only 34% of patients were free from inflammation. No other histological details were given.

In another study, 264 patients with distal active UC were treated with 5-ASA foam or liquid enema<sup>[57]</sup>. Histological assessment (no details were given), performed after 4 wk, showed similar improvement in both groups (46% generically defined remission in the foam group and 50% in the enema group).

An 8-wk double-blind multicenter trial compared three doses of 5-ASA (1.5, 3 and 4.5 g/d) in 321 patients with active UC<sup>[58]</sup>. Colonic biopsies were obtained at baseline and at the end of the study, and were assessed according to a previously described scoring system<sup>[29]</sup>, considering histological improvement as reduction of at least one point of the histological activity index. At the end of study period, histological improvement was documented in 42% patients in the 1.5 g group, 65% of the 3 g group, and 63% of the 4.5 g group. Once again, no details on histological assessment were given.

Other authors compared the effects of slow-release (MMx) mesalazine with topical 5-ASA in 79 patients with active left-side UC<sup>[59]</sup>. The tissue inflammatory response was evaluated according a previously described score<sup>[26]</sup>, and graded as follows: grade 1, normal mucosa; grade 2, enhanced glands with intraepithelial granulocytes. In the stroma, enhancement beyond normal of lymphocytes,

plasma cells, or eosinophils (slight inflammation); grade 3, goblet cells depletion, loss of tubular parallelism, reduced mucin production in some glands, intraepithelial granulocytes, marked increase of inflammatory cells in the stroma (intermediate inflammation); grade 4, marked gland and mucosal atrophy, evident crypt abscesses and pus on the surface, massive increase of acute inflammatory cells and follicle formation in deeper cell layers (severe inflammation); and grade 5, ulceration with pus, gland and mucosal atrophy, crypt abscesses, extensive stromal inflammation, and deep follicles (fulminant inflammation). Histological remission (defined generically according to the above score) was obtained in only 15% patients of the MMx group and in 8.0% of those receiving enemas. No other histological details were given.

A more recent study compared two different scheduling dosages (3 g *oid* or 1 g *tid*) of mesalazine granules in 380 UC patients<sup>[60]</sup>. Biopsy samples were obtained at the start and the end of the trial, and were scored according to previously published criteria<sup>[29]</sup>, with the total histological index based on the more severely affected colonic segment. Histological remission (grade 0), observed in 35% patients in the *oid* group and in 41% of the *tid* group, did not show significant differences between the two regimens.

Overall, the available evidence for salicylates in histological healing of UC patients suggests that an improvement may be obtained in 30%-60% of patients (obviously depending on the formulation and the dose scheduling), but the actual healing rate is lower (10%-30%).

### Immunomodulators

There are still only a few data on histological healing with immunomodulatory agents. A study conducted in 32 patients with refractory active UC<sup>[61]</sup> showed that after 6 mo azathioprine treatment, 78% of patients in remission were free of histological inflammation graded according to previously described criteria<sup>[23]</sup>. However, after a median of 4 years follow-up, histological relapse was found in almost 90% of these patients. No histological details were given.

Another study compared the effects of intravenous cyclosporine A to those of methylprednisolone in 30 patients with severe UC<sup>[62]</sup>. Histological assessment, carried out according to a previously described scoring system<sup>[33]</sup> (Table 3), was done at baseline, and after 7 and 30 d therapy; after 1 wk no effects were seen, and only after 1 mo therapy was there a significant decrease in inflammatory cells, and severity of epithelial damage was similarly observed in both groups, although only small changes in the architectural mucosal disturbances were seen. Overall, it seems however that these agents have a beneficial effect on mucosal histology.

### Biological agents

Concerning biological therapy, although anti-tumor necrosis factor (TNF)- $\alpha$  therapy can lead to endoscopically assessed mucosal healing in patients with UC<sup>[63,64]</sup>,

**Table 3** Histological scoring system for ulcerative colitis and Crohn's disease

Ulcerative colitis	
Grade 0	Structural (architectural change)
0	No abnormality
0.1	Mild abnormality
0.2	Mild or moderate diffuse or multifocal abnormalities
0.3	Severe diffuse or multifocal abnormalities
Grade 1	Chronic inflammatory infiltrate
1	No increase
1.1	Mild but unequivocal increase
1.2	Moderate increase
1.3	Marked increase
Grade 2	Lamina propria neutrophils and eosinophils
2A. Eosinophils	
2A.0	No increase
2A.1	Mild but unequivocal increase
2A.2	Moderate increase
2A.3	Marked increase
2B. Neutrophils	
2B.0	None
2B.1	Mild but unequivocal increase
2B.2	Moderate increase
2B.3	Marked increase
Grade 3	Neutrophils in epithelium
3	None
3.1	< 5% crypt involved
3.2	< 50% crypt involved
3.3	> 50% crypt involved
Grade 4	Crypt destruction
4	None
4.1	Probable-local excess of neutrophils in part of crypt
4.2	Probable-marked attenuation
4.3	Unequivocal crypt destruction
Grade 5	Erosion or ulceration
5	No erosion, ulceration, or granulation tissue
5.1	Recovering epithelium plus adjacent inflammation
5.2	Probable erosion-focally stripped
5.3	Unequivocal erosion
5.4	Ulcer or granulation tissue
Crohn's disease	
Epithelial damage	
0	Normal
1	Focal pathology
2	Extensive pathology
Architectural changes	
0	Normal
1	Moderately disturbed (< 50%)
2	Severely disturbed (> 50%)
Infiltration of mononuclear cells in the lamina propria	
0	Normal
1	Moderate increase
2	Severe increase
Infiltration of polymorphonuclear cells in the lamina propria	
0	Normal
1	Moderate increase
2	Severe increase
Polymorphonuclear cells in the epithelium	
1	In surface epithelium
2	Cryptitis
3	Crypt abscess
Presence of erosions and/or ulcers	
0	No
1	Yes
Presence of granuloma	
0	No
1	Yes
No. of biopsy specimens affected	
0	None (0 of 6)
1	< 33% (1 or 2 of 6)
2	33%-66% (3 or 4 of 6)
3	> 66% (5 or 6 of 6)

Each topic scored independently. Moderate increase, up to twice the number of cells that can normally be expected; severe increase, more than twice the normal number of cells.

histological data are still scarce. A 10-wk study conducted on nine moderate to severe UC patients<sup>[65]</sup>, using a score that included polymorphonuclear infiltration of the epithelium and lamina propria, crypt abscesses, loss of glandular parallelism, crypt shortening and/or ramification, mucus epithelial depletion, involvement of muscularis mucosae and/or submucosa. Each histological variable was scored from 0 (normal) to 1 (mild), 2 (moderate) and 3 (severe). In addition, the total number of neutrophils, lymphocytes, and plasma cells in the lamina propria was counted in five high-power fields. At week 10, histological score significantly decreased only in responders (67% of patients), but normal architecture was observed only in 33% of these patients. The histological improvement was mainly due to a decrease in neutrophils associated with restoration of normal crypt architecture and mucus content in epithelial cells. No significant reduction of mononuclear cell infiltration was observed in responders and non-responders.

Another study took into account the ultrastructural features of the colon in seven patients with UC refractory to standard treatment, before and after 2 wk treatment with infliximab<sup>[66]</sup>. Before treatment, severe alterations of the epithelium were present, such as microvilli depletion, shattering of epithelial junctions, cytoplasmic vacuolization, dilatation of the endoplasmic reticulum, pycnotic nuclei, altered structure of mitochondria and Golgi complexes, in addition to rarefaction of the goblet cells with abnormal mucus formation and secretion. The chorion showed structural alteration of component cells, obstructed capillaries, erythrocyte extravasation, and many plasmocytes and neutrophils. After infliximab, there was improvement in morphology and function of the epithelial organelles, rich mucus secretion, and recovery of the chorionic components.

### Miscellaneous

D'Ovidio *et al*<sup>[67]</sup> have evaluated the effects of granulocyte-monocyte apheresis in 12 patients with mild to moderate UC refractory to therapy/steroid dependent. After 6 wk, complete histological remission, defined as grade 1 according to the score of Florén *et al*<sup>[26]</sup>, was obtained in six (50%) patients, and histological improvement in five (42%) patients. Once again, no further details on histology were given. In general, it seems that various drugs can lead to microscopic healing and a normal mucosa in a minority of patients (with a maximum of approximately 30%).

## HISTOLOGICAL HEALING IN CD

### Corticosteroids

These agents are frequently employed for treatment of disease flares; however, clinical and endoscopic data demonstrate that mucosal healing in CD is unlikely (less than one third of treated patients) after steroid treatment<sup>[68]</sup>. Moreover, there have been few studies evaluating histological healing in CD after steroid treatment.

In a study comparing 1 year treatment with budeso-



nide or azathioprine in 77 CD patients, histological assessment was carried out with a previously developed score<sup>[37]</sup> (Table 3). At the end of the study, the histological score fell significantly versus baseline only in the azathioprine group and was significantly lower than in the budesonide group at the end of the study<sup>[69]</sup>. This improvement was observed for all acute parameters (epithelial damage, acute lamina propria inflammatory cell infiltration, erosions and/or ulcers) irrespective of disease location, whereas glandular architecture and the presence (but not the degree) of chronic inflammation were not influenced by azathioprine treatment.

A 2-wk regimen of 20 mg oral prednisone showed, in individual biopsies of 30 CD patients undergoing surveillance colonoscopy, a decrease in the overall histological activity (assessed by a previously reported scoring system<sup>[33]</sup>) compared to placebo, whereas no significant differences were found with respect to the overall severity of inflammation<sup>[70]</sup>. No other details on histological assessment were given.

### Immunomodulators

These agent represent to date the cornerstone of treatment in CD, thus researchers' interest has given rise to a few studies aimed at evaluating their effects on histological healing as well.

**Azathioprine:** A first study assessed the effect of 6 mo of therapy in 19 patients with severe postoperative recurrent CD<sup>[71]</sup>. Histological evaluation was done in seven patients (four with complete mucosal healing and three with near-complete healing at endoscopy). Only in patients with complete healing did comparison of the biopsy specimens taken before and after azathioprine show persistent mucosal architectural changes with complete disappearance of the inflammatory infiltrate. The same authors then reported pre/post-therapy data on 20 CD patients with colitis-ileocolitis taking azathioprine for at least 9 mo<sup>[72]</sup>, using a histological disease severity score<sup>[37]</sup>. A decrease in histological score paralleled endoscopic healing, even though it was not as complete as shown by endoscopy. In patients with endoscopic complete healing, the colon showed a global decrease in histological score from 10 to 3 and inflammatory score from 5 to 2; in the ileum, global histological score decreased from 7 to 2 and inflammatory score decreased from 2 to 0. No significant changes were documented in patients without endoscopic healing while taking azathioprine. The study of Mantzaris *et al.*<sup>[69]</sup>, comparing azathioprine with budesonide, has been discussed above.

**Methotrexate:** Methotrexate has been even less frequently investigated in this setting. A pilot study evaluated the effects of adding this agent to standard treatment (steroids, mesalazine) in 14 CD patients with refractory disease<sup>[73]</sup>; four patients (28.5%) were reported to have normal histology at 12 wk. Another study compared the effects of methotrexate with those of azathioprine and

infliximab in 40 patients with CD<sup>[74]</sup>. After 3 mo treatment, the scoring of microscopic activity<sup>[37]</sup> was similar in the three groups. No further histological data or data on histological healing were given.

### Biological agents

A few studies have been carried out on biological agents. D'Haens *et al.*<sup>[75]</sup> investigated histological healing in 30 CD patients treated with infliximab in a placebo-controlled trial. After 4 wk intravenous administration of 5, 10 or 20 mg/kg infliximab or placebo as a single infusion, inflammatory infiltrate disappeared only in infliximab-treated patients but architectural changes persisted in most of them. Another study from the same group evaluated the effects of a single infusion of infliximab in 15 patients with steroid-refractory CD<sup>[76]</sup>. Histological activity scores, assessed 4 wk after infusion showed, compared to placebo, a significant decrease mainly due to the reduction of inflammatory activity. Immunohistochemical assessment also revealed a decrease in inflammatory features due to global reduction of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes and CD68<sup>+</sup> mononuclear cells.

A follow-up study conducted in CD patients evaluated histological improvement by a previously described scoring system<sup>[37]</sup> after 54 wk of treatment with infliximab, and showed that histological mucosal healing was associated with a consistent decrease in the expression of inflammatory markers<sup>[77]</sup>. The use of etanercept (another anti-TNF- $\alpha$  agent) in 10 patients with refractory active CD did not result in any significant histological improvement after 2 wk of therapy<sup>[78]</sup>.

### Miscellaneous

Yamamoto *et al.*<sup>[79]</sup> evaluated 28 patients with active CD, treated with an elemental diet for 4 wk. Histology was evaluated by a previously described scoring system<sup>[80]</sup>, taking into consideration enterocyte appearance, extent of crypt inflammation, and the intensity of mononuclear and neutrophilic lamina propria inflammation. Each feature was given a score of 0-3, depending on the severity of the abnormality. The scores were added, and a total score was calculated. Grade 0 (no inflammation) was assigned a total score of 0 or 1, grade 1 (mild) was scored 2-4, grade 2 (moderate) was scored 5-8, and grade 3 (severe) was scored 9-12. Histological healing was defined as grade 0, and histological improvement (including healing) was defined as a decrease of at least 1 grade. At the end of treatment, histological healing and improvement rates were 19% and 54% in the terminal ileum and 20% and 55% in the large bowel, respectively.

A retrospective study carried out in 39 patients with refractory severe colonic CD evaluated the effect of anti-*Mycobacterium avium* ss *paratuberculosis* therapy<sup>[81]</sup>. Histological acute and chronic ileitis or colitis were recorded as the most severe inflammation found in available biopsies, and were generically reported by a pathologist as ranging from inactive chronic inflammation to mild, moderate, and severe active inflammation. After an aver-

age 3 years of therapy, 15/39 (38.5%) patients showed a marked reduction of acute and chronic inflammatory infiltrates; however, only in six (15.4%) patients was normalization of histological variables observed. No other histological variables or details were given.

## HISTOLOGICAL HEALING IN IBD: WHERE ARE WE, AND WHERE ARE WE GOING?

Although mucosal healing has been associated with positive outcomes in IBD, most of the supporting data are retrospective and largely based on endoscopic assessment; in addition, it is not clear whether complete mucosal healing produces better outcomes than partial healing<sup>[82]</sup>. It has been stated that, in clinical practice, evaluation of mucosal healing (again, by an endoscopic point of view) should be considered in patients with persistent symptoms despite adequate therapy and when treatment discontinuation is being considered<sup>[83]</sup>.

However, it must be stressed that histological assessment of mucosal healing, the only way to establish in an objective manner that the mucosa has reverted to a normal state, is at present not recommended as a primary end point for therapeutic trials because of the lack of a standardized approach<sup>[5]</sup>. Thus, it is unknown whether patients who achieve and maintain deep remission may stop treatment without having further/future problems<sup>[84]</sup>.

As reported above, true histological healing may be grossly obtained in only about 30% of treated patients; this may justify the high (20%-40%) loss of response to even newer therapeutic approaches<sup>[85]</sup>. A recent study has shown that adding histological and mucosal gene panel assessment may predict the lack of response to infliximab (directed against TNF- $\alpha$ ) treatment in patients with UC or Crohn's colitis, whereas no such predictive gene set could be identified for ileal disease<sup>[86,87]</sup>.

Therefore, adding histological healing as an endpoint, at least in clinical trials, would substantially improve the evaluation of the actual effectiveness of a given treatment. Histological assessment is cheap and widely available. The features of disease activity are known. They include the presence of neutrophils in the lamina propria and the epithelium, epithelial cell damage (loss of cells, mucin depletion, cryptitis, crypt abscesses, erosion), and an increase in lymphocytes and plasma cells. Some of these features such as the presence of neutrophils can reliably be evaluated with good interobserver agreement. In addition, histology could help the management of the patient. Some histological variables are indeed associated with relapse. They include persistence of neutrophils, basal plasmacytosis and persistence of eosinophils in the lamina propria<sup>[29,88,89]</sup>. Furthermore, microscopic activity may be related to the development of dysplasia<sup>[90]</sup>.

We realize that adding data on histological healing to investigations and trials on IBD patients might be a relatively difficult goal to achieve, and that such an approach would increase the investigators' burden. Furthermore, there are several unresolved issues. It has been shown that UC may become a discontinuous inflammatory pro-

cess during its natural course or following medical treatment. This means that several biopsies would be needed for an adequate microscopic evaluation; the optimal number of samples has however not yet been determined. Although routine HE staining is a good tool for identification of neutrophils, epithelial damage and even eosinophils as markers of active disease, it is less appropriate for the evaluation of lymphocytes. The presence of lymphocytes can be determined adequately but the activation status is beyond the scope of routine histology and requires immunohistochemical staining. Yet, the application of this technique and the markers needed are not yet well determined.

## CONCLUSION

In conclusion, it appears to us that histological healing should be considered as an end point in the treatment of IBD patients, especially for UC. Therefore, additional studies looking for optimal sampling, standardizing scores and features should be performed.

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## Colorectal cancer mortality in Hong Kong of China, Japan, South Korea, and Singapore

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### Abstract

To clarify the trend in colorectal cancer mortality in Asian countries. We analyzed the colorectal cancer mortality in four Asian countries using the World Health Organization mortality database and the Korea National Statistics Office database. The annual age-standardized rates and truncated rates for the three age groups (30-49, 50-69 and  $\geq 70$  years) for Hong Kong of China (1969-2009), Japan (1955-2009), South Korea (1985-2006), and Singapore (1966-2009) were estimated. A joinpoint regression model was used to detect significant trends in mortality rates. Colorectal cancer mortality in men started to decrease in 1992 in Japan followed by Singapore and Hong Kong of China in 1995. The mortality rates in women started to decrease in 1980 in Singapore, followed by Hong Kong of China

and Japan in 1996. In all countries and both genders, except for women in Singapore, the decrease in mortality began in the younger age groups. The colorectal cancer mortality in the four studied Asian countries has started to decrease, and the decrease occurred first in the younger age groups.

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**Key words:** Colorectal cancer; Mortality; Joinpoint regression; Trends; Early detection of cancer; Mass screening

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### INTRODUCTION

Colorectal cancer is responsible for 8% of all cancer deaths worldwide, with an estimated 608 000 deaths annually, and it is ranked as the fourth most common cause of death from cancer<sup>[1]</sup>. It has been estimated that the numbers of deaths due to colorectal cancer will reach approximately 376 700 by 2020 in Asia<sup>[1]</sup>.

A decline in colorectal cancer mortality has been observed in most western and northern European countries<sup>[2]</sup> and the United States<sup>[3]</sup>. Among Asian countries, the mortality started to decrease in the early 1990s in Japan<sup>[4,5]</sup>. The trends in colorectal mortality differ by geographic region, sex, and age group<sup>[6]</sup>. In this review, we analyzed the trends in colorectal cancer mortality in 4 Asian countries stratified by sex and age groups.

### DATA ANALYSIS

Colorectal cancer mortality data were extracted from the World Health Organization (WHO) mortality data-

base<sup>[7]</sup>. The 3 countries or regions with data available for the longest period were Japan (since 1955), Singapore (since 1966) and Hong Kong of China (since 1969) were included in the analysis. In addition, Korean data were available from the WHO mortality database for 1985 to 2006 and from the Korea National Statistics Office for 2007 to 2010 and were included in the study<sup>[8]</sup>. The annual age-standardized rates (ASR) and the truncated rates for the 3 age groups (30-49, 50-69 and  $\geq 70$  years) were estimated using the world standard population.

The trends in colorectal cancer mortality were tested using joinpoint regression models, using Joinpoint software version 3.5.3. The software was developed by the Surveillance Research Program of the United States National Cancer Institute and is based on the Poisson assumption<sup>[9]</sup>. A maximum of 3 joinpoints was allowed, and the default settings were used.

Table 1 shows the age-adjusted mortality rates for colorectal cancer in the 4 countries. Singapore showed the highest female mortality rates of the 4 countries from 1966 to 2009. Singapore also showed the highest male mortality during the same period, except for 2005, when males in Hong Kong of China showed a slightly higher rate. Korean men and women showed the lowest rates among the 4 countries.

Table 2 shows the results for the joinpoint regression analyses. Colorectal cancer mortality in men started to decrease in 1992 in Japan, followed by Singapore and Hong Kong of China in 1995. The mortality rates in women started to decrease in 1980 in Singapore, followed by Hong Kong of China and Japan in 1996. In South Korea, the mortality rates plateaued in 2002 in men and started to decrease in 2004 in women.

In men, the decline in mortality started for younger age groups first. In Japan, mortality started to decrease in 1977 for the 30-49 years age group, in 1995 for the 50-69 years age group, and in 1998 for the 70 and older age group (Table 2). A similar trend was observed for Hong Kong of China, Singapore and South Korea. Similarly, a significant decrease in APC was first observed for younger age groups in Hong Kong of China, Japan and South Korea in women. For example, in Hong Kong of China, female mortality began to decrease in 1992 for the 30-49 years age group, in 1994 for the 50-69 years age group, and in 2002 for the 70 years and older age group. Whereas, for women in Singapore, the decrease began in 1980 in the 50-69 years age group, which was 4 years earlier than among the 30-49 years age group (Table 2). Figure 1 shows the trends in colorectal cancer mortality rates for the 3 age groups in Hong Kong of China, Japan, South Korea, and Singapore between 1955 and 2010.

## DISCUSSION

Colorectal cancer mortality in the European Union has declined since the early 1980s. These decreases were observed in most western and northern European countries, whereas a persistent excess in mortality was observed in Hungary and the Czech Republic<sup>[6]</sup>. In the United States, colorectal cancer mortality rates in white men began to

decline in 1978<sup>[10]</sup>. The APC for white men was -0.6% between 1973 and 1978 and -2.0% in 1986<sup>[10]</sup>. The decline in the mortality of white women was more rapid, with an APC of -2.1% between 1973 and 1997<sup>[10]</sup>.

In Asia, a reduction in colorectal cancer mortality has been observed for economically advanced regions, such as Japan, Hong Kong of China, Singapore, and more recently, in South Korea. Notably, the decline started in younger age groups. A more favorable mortality trend and a consequent widening of the survival gap between the elderly and middle age groups were observed in Europe<sup>[2,6,11]</sup>.

In contrast, the incidence rates for colorectal cancer in these countries have increased, except for men and women in Japan and women in Hong Kong of China<sup>[4,12-14]</sup>. Singapore experienced a sharp increase in the colorectal cancer incidence between 1968 and 2002, particularly among older men<sup>[12]</sup>. In Hong Kong of China, the ASR peaked in 1994 and has since declined in women, whereas the ASR progressively increased in men<sup>[14]</sup>. The increase was notable among men above 60 years old and women above 70 years old<sup>[14]</sup>. Similarly, the APCs for the incidence rate of colorectal cancer was prominent among older Korean men and women between 1999 and 2009<sup>[13]</sup>. Japan is the only Asian country in which the incidence rates for colorectal cancer have decreased in both men and women<sup>[4]</sup>. The Osaka Cancer Registry data showed that the overall colorectal cancer incidence in women has decreased since 1995. In men, the rate has been stabilized since 1996<sup>[9]</sup>.

Changes in risk factors, particularly those related to lifestyle, have been suspected as main contributors to the colorectal cancer increase. Among modifiable lifestyle factors, alcohol consumption, obesity, cigarette smoking, and dietary habits (*e.g.*, red meat and processed meat consumption) have been associated with colorectal cancer risk<sup>[15,16]</sup>. In a study for population-attributable fractions of cancer in Japan, 31%-33% of the colorectal cancer incidence or mortality was explained by known preventable risk factors, such as alcohol consumption, cigarette smoking, obesity, and physical inactivity<sup>[17]</sup> when men and women combined. Among them, alcohol consumption was attributed for the greatest portion, followed by cigarette smoking and obesity. In a Chinese study, the population-attributable fraction of known preventable risk factors for cancer death was 14.6% for colon cancer and 2.2% for rectal cancer<sup>[18]</sup>. Alcohol consumption was accountable for 32.9% of male colorectal cancer and 2.1% of female colorectal cancer in Japan<sup>[17]</sup>. In China, alcohol consumption was accountable for 2.1% and 0.2% of colorectal cancer cases in men and women, respectively<sup>[19]</sup>. The prevalence and amount of alcohol consumption in these countries and South Korea have not decreased during the last few decades<sup>[17,18,20]</sup>. However, the prevalence of obesity, particularly in men, and a sedentary lifestyle has increased in Hong Kong of China, South Korea, and Japan<sup>[14,17,21]</sup>. Fortunately, the prevalence of cigarette smoking has declined in South Korea, Japan, and China<sup>[17,18,20]</sup>. These changes may explain the transition in the colorectal cancer epidemiology in these Asian countries.

**Table 1** Colorectal cancer mortality rates in Hong Kong of China, Japan, South Korea and Singapore, 1955-2010 (age-standardized mortality rates per 100 000)

Country or area	Period	Men				Women			
		1955	1975	1995	2005	1955	1975	1995	2005
Hong Kong (China)	1969-2009	-	29.7	40.7	37.8	-	18.8	26.1	23.4
Japan	1955-2009	14.5	23.8	38.2	34.8	12.5	18.3	22.3	20.8
South Korea	1985-2010	-	-	8.6	30.5	-	-	11.8	17.2
Singapore	1966-2009	-	37.2	52.1	36.8	-	26.1	31.7	27.5

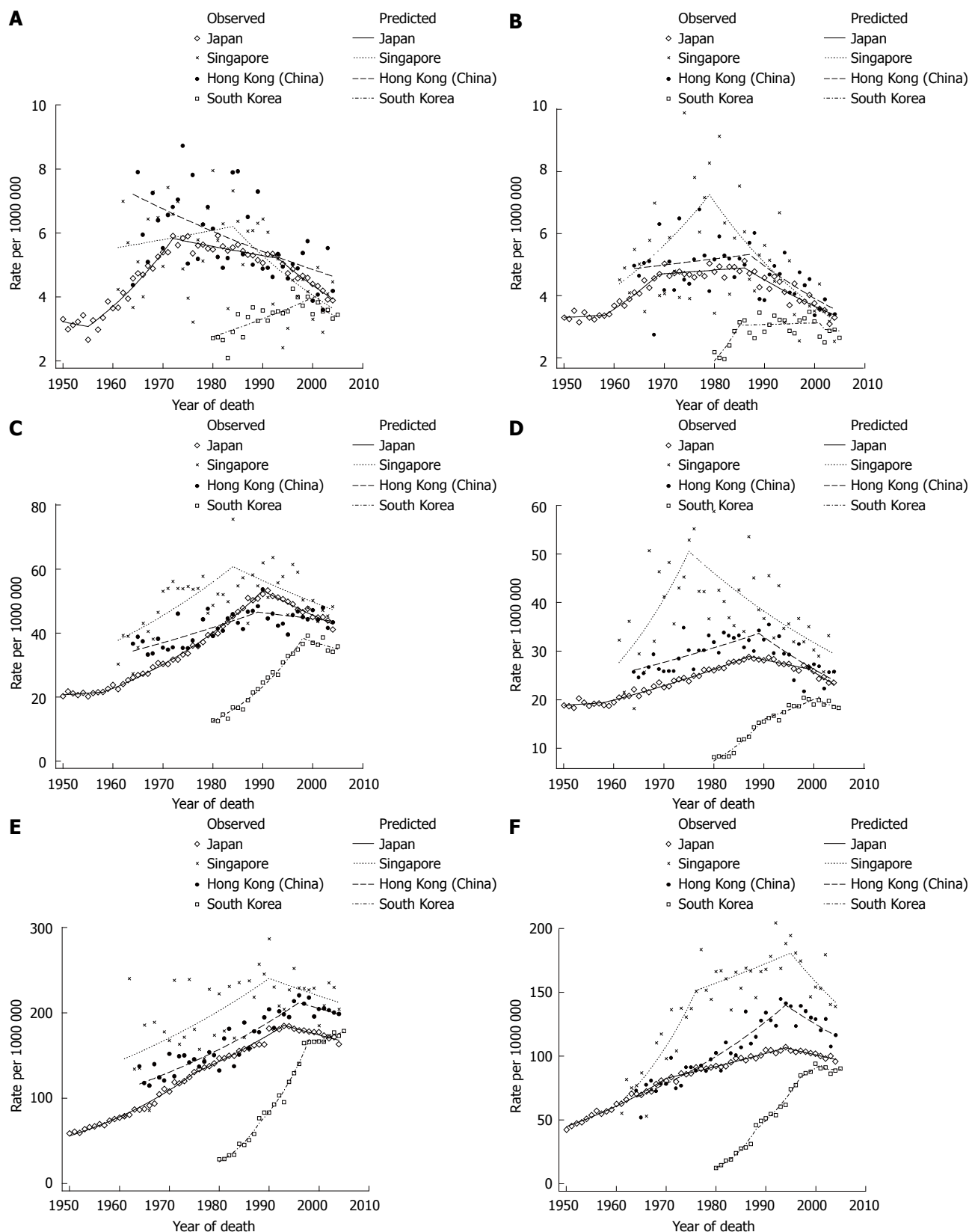
**Table 2** Joinpoint analysis for colorectal cancer mortality at all ages and at age 30-49, 50-69 and  $\geq 70$  year in Hong Kong of China, Japan, South Korea and Singapore, 1955-2010

	Trend 1		Trend 2		Trend 3		Trend 4	
	Year	APC	Year	APC	Year	APC	Year	APC
Men								
All ages								
Hong Kong (China)	1969-1995	1.3 <sup>1</sup>	1995-2009	-0.1	1992-2004	-0.4 <sup>1</sup>	2004-2009	-2.21
Japan	1955-1975	2.0 <sup>1</sup>	1975-1992	1.2 <sup>1</sup>				
South Korea	1985-2002	7.9 <sup>1</sup>	2002-2010	0.3				
Singapore	1966-1995	1.4 <sup>1</sup>	1995-2009	-1.5 <sup>1</sup>				
30-49 yr								
Hong Kong (China)	1969-2009	-1.1 <sup>1</sup>	1960-1977	3.8 <sup>1</sup>	1977-1998	-0.5 <sup>1</sup>	1998-2009	-2.51
Japan	1955-1960	-1.0						
South Korea	1985-2002	2.5 <sup>1</sup>						
Singapore	1966-1989	0.5						
50-69 yr								
Hong Kong (China)	1969-1994	1.2 <sup>1</sup>	1994-2009	-0.4	1995-2009	-1.6 <sup>1</sup>		
Japan	1955-1963	0.4	1963-1995	2.9 <sup>1</sup>				
South Korea	1985-2003	6.5 <sup>1</sup>	2003-2010	-1.4 <sup>1</sup>				
Singapore	1966-1989	2.1 <sup>1</sup>	1989-2009	-1.2 <sup>1</sup>				
≥ 70 yr								
Hong Kong (China)	1969-2001	1.9 <sup>1</sup>	2001-2009	-0.8	1998-2009	-0.9 <sup>1</sup>	2002-2010	1.11
Japan	1955-1980	3.4 <sup>1</sup>	1980-1998	2.0 <sup>1</sup>				
South Korea	1985-1994	14.0 <sup>1</sup>	1994-1998	5.9				
Singapore	1966-1995	1.7 <sup>1</sup>	1995-2009	-0.9				
Women								
All ages								
Hong Kong (China)	1969-1996	1.5 <sup>1</sup>	1996-2009	-1.7 <sup>1</sup>	1983-1996	0.9 <sup>1</sup>	1996-2009	-0.71
Japan	1955-1960	14.3 <sup>1</sup>	1960-1983	2.9 <sup>1</sup>				
South Korea	1985-1994	9.9 <sup>1</sup>	1994-2004	4.3 <sup>1</sup>				
Singapore	1966-1980	4.7 <sup>1</sup>	1980-1998	-0.2				
30-49 yr								
Hong Kong (China)	1969-1992	0.4	1992-2009	-2.4 <sup>1</sup>	1974-1991	0.3	1991-2009	-2.11
Japan	1955-1963	0.2	1963-1974	3.1 <sup>1</sup>				
South Korea	1985-1991	8.8 <sup>1</sup>	1991-2010	-0.6				
Singapore	1966-1984	2.8	1984-2009	-3.2 <sup>1</sup>				
50-69 yr								
Hong Kong (China)	1969-1994	1.0 <sup>1</sup>	1994-2009	-2.3 <sup>1</sup>	1992-2004	-0.8 <sup>1</sup>	2004-2009	-2.51
Japan	1955-1963	0.4	1963-1992	1.4 <sup>1</sup>				
South Korea	1985-1994	8.3 <sup>1</sup>	1994-2003	3.3 <sup>1</sup>				
Singapore	1966-1980	4.4 <sup>1</sup>	1980-2009	-1.8 <sup>1</sup>				
≥ 70 yr								
Hong Kong (China)	1969-2002	2.5 <sup>1</sup>	2002-2009	-1.9 <sup>1</sup>	1999-2009	-0.9 <sup>1</sup>		
Japan	1955-1975	3.1 <sup>1</sup>	1975-1999	1.1 <sup>1</sup>				
South Korea	1985-1993	16.9 <sup>1</sup>	1993-2003	7.7 <sup>1</sup>				
Singapore	1966-1981	5.8 <sup>1</sup>	1981-2000	0.9 <sup>1</sup>				

<sup>1</sup>*P* < 0.05 vs the younger age groups. APC: Annual percent change.

A rapid increase in the incidence among older age groups may reflect the accumulated exposure to risk factors<sup>[22]</sup>. In contrast, a major contributor to the mortality reduction for colorectal cancer in the younger generation is the adaptation of screening programs. In Japan, a

colorectal cancer screening program using a fecal occult blood test (FOBT) has been in place since 1992 under the Health Services Law for the Aged<sup>[23]</sup>. Colorectal cancer screening programs were introduced as a part of the National Cancer Screening Program for Medical Aid



**Figure 1** Trends in age-standardized colorectal cancer mortality rates per 100 000 in Hong Kong of China, Japan, South Korea and Singapore, 1955-2010. A: Men 30-49 yr; B: Men 50-69 yr; C: Men  $\geq$  70 yr; D: Women 30-49 yr; E: Women 50-69 yr; F: Women  $\geq$  70 yr.

recipients and National Health Insurance beneficiaries in the lower income bracket in 2004 in South Korea<sup>[24]</sup>. The FOBT is provided free of charge as a primary modality for men and women aged 50 years or older. FOBT-pos-

itive individuals were provided follow up by either colonoscopy or a double-contrast barium enema<sup>[24]</sup>. According to the Korean National Cancer Screening Survey, which covers both organized and opportunistic cancer screen-



ing programs, the lifetime screening rates for colorectal cancer were 25.3% in 2004 and 54.2% in 2010<sup>[25]</sup>. Although colorectal cancer screening for the average-risk population is recommended to start at 50 years of age, a national screening program is not available in Singapore<sup>[26]</sup>. However, compliance with opportunistic screening in Singapore was reasonably high<sup>[26]</sup>.

## CONCLUSION

In Hong Kong of China, Japan, South Korea and Singapore in which economic development and the westernized lifestyle were adopted early, colorectal cancer mortality has started to decrease. The decline or stabilization of mortality occurred the earliest in younger age groups and in women. The most important contributor to the decline in mortality is the introduction of colorectal cancer screening programs, although the role of the transition of lifestyle risk factors needs to be addressed.

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## Molecular mechanisms of chemopreventive phytochemicals against gastroenterological cancer development

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are regarded as promising chemopreventive agents. Hence, regular consumption of these natural bioactive compounds found in foods can contribute to prevention, suppression, and/or delay of gastroenterological cancer development. In this review, we will summarize natural phytochemicals possessing potential antioxidant and/or anti-inflammatory and anti-carcinogenic activities, which are exerted by regulating or targeting specific molecules against gastroenterological cancers, including esophageal, gastric and colon cancers.

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**Key words:** Curcumin; Resveratrol; (-)-Epigallocatechin gallate; Isothiocyanates; Sulforaphane; Gastroenterological cancers; Molecular target

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### Abstract

Cancer is one of the leading causes of death worldwide. Commonly used cancer treatments, including chemotherapy and radiation therapy, often have side effects and a complete cure is sometimes impossible. Therefore, prevention, suppression, and/or delaying the onset of the disease are important. The onset of gastroenterological cancers is closely associated with an individual's lifestyle. Thus, changing lifestyle, specifically the consumption of fruits and vegetables, can help to protect against the development of gastroenterological cancers. In particular, naturally occurring bioactive compounds, including curcumin, resveratrol, isothiocyanates, (-)-epigallocatechin gallate and sulforaphane,

### INTRODUCTION

Cancer is a leading cause of death worldwide. Surgery, chemotherapy and radiation therapy are commonly used cancer treatments. However, these can cause a number of side effects, and complete cure is often infeasible for most patients suffering from specific cancers. Epidemiological studies have suggested that regular consumption of vegetables, fruits, red wine and tea is associated with lower incidences of many chronic diseases, including cancers<sup>[1-4]</sup>. A recent review reported that natural bioactive compounds found in various foods can activate or deactivate molecular signaling cascades by targeting small molecules in cancer cells<sup>[5]</sup>. A number of natural phytochemicals, including isoflavones, gingerol, (-)-epi-

gallicocatechin gallate (EGCG), quercetin, resveratrol, and curcumin have been identified to be chemopreventive and their significant health benefits are an active field of research<sup>[5]</sup>. In particular, gastroenterological cancers are closely associated with lower consumption of fruits or vegetables. Therefore, in this review, we will summarize the natural phytochemicals possessing potential antioxidant and/or anti-inflammatory and anti-carcinogenic activities, which act by regulating or targeting specific molecules against gastroenterological cancers, including esophageal, gastric and colon.

## PHYTOCHEMICALS IN ESOPHAGEAL CANCER

Esophageal cancer is the eighth most common cancer and is the sixth most common cause of cancer-related deaths<sup>[6]</sup>. Due to the lack of symptoms, individuals are rarely aware of their condition until the metastatic stages of the disease<sup>[7]</sup>. Despite developments in current cancer treatments, including chemotherapy, radiation therapy, and surgery (esophagogastric resection), patients with esophageal adenocarcinoma are not often cured of the disease<sup>[8]</sup>. Statistical analysis for the past 5 years shows that Americans and Europeans with esophageal cancer have relatively low survival rates, 10%-15% and 10% respectively<sup>[9]</sup>. Moreover, this suggests that the esophageal tumors are resistant to regular therapies and, thus, alternative strategies for the treatment and/or prevention of esophageal cancer are required.

Major risk factors for esophageal cancer are chewing and smoking tobacco, drinking alcoholic beverages<sup>[10]</sup>, low consumption of fruits and vegetables<sup>[1]</sup> and consumption of salt-cured, salt-pickled, and moldy foods<sup>[11]</sup>. Therefore, quitting cigarette smoking, reducing alcohol consumption, increasing fruit and vegetable consumption, and avoiding foods containing nitrosamines and nitrosamine precursors are critical for the prevention of this disease. In addition to such lifestyle changes, identifying foods or food constituents that can help to prevent, suppress, and/or delay the onset of esophageal cancer is essential<sup>[12,13]</sup>. Recent attention has focused on the beneficial actions of natural phytochemicals, such as isothiocyanates, curcumin, and resveratrol, against esophageal cancer (Table 1). Although the underlying molecular mechanisms have not been fully understood, such natural chemicals are known to protect against disease progression by targeting specific proteins.

### Isothiocyanates

Isothiocyanates are naturally occurring phytochemicals in cruciferous vegetables, including Chinese watercress, cabbage, Brussels sprouts, turnips and cauliflower<sup>[14]</sup>. In the gastrointestinal tract, isothiocyanates are released from their precursor *via* hydrolysis catalyzed by myrosinase. Of its metabolites, phenethyl isothiocyanate (PEITC) has been reported to be rapidly absorbed and distributed in mice following oral administration<sup>[15]</sup>. Studies have

demonstrated that PEITC ( $> 1.0 \mu\text{mol/g}$  diet) protects against esophageal cancer by inhibiting tumor incidence and multiplicity in rats treated with N-nitrosobenzylmethylaniline (NMBA), the most potent inducer of esophageal tumors and is commonly used to study the pathogenesis of esophageal cancer<sup>[16]</sup>.

Several studies of the molecular mechanism whereby PEITC inhibits NMBA-induced esophageal tumorigenesis have revealed that PEITC suppresses the activity of cytochrome P450 enzymes in rats with NMBA-induced esophageal cancer<sup>[11,17,18]</sup>, and also inhibits DNA methylation by inhibiting the formation of the pro-mutagenic adduct O<sup>6</sup>-methylguanine in rat esophageal DNA<sup>[16]</sup>. Significant correlations between DNA adduct formation and tumor multiplicity have been observed in rat lungs as well as esophagi<sup>[18]</sup>, indicating that DNA adduct formation probably contributes to tumor incidence and multiplicity. Collectively, PEITC is likely to have anti-carcinogenic activity *via* regulation of P450 enzyme activity and inhibition of DNA damage, contributing to the prevention of esophageal cancer. However, no direct target has been identified. Hence, future investigation is needed to elucidate the molecular target(s) of isothiocyanates or their metabolites in the prevention and/or treatment of esophageal cancer.

### EGCG

Polyphenols are major components of tea. One-third of the dry weight of green or black tea is composed of polyphenols, which have powerful antioxidant and anti-inflammatory potential<sup>[19]</sup>. Wang *et al.*<sup>[20]</sup> reported that both decaffeinated green and black tea consumption reduced esophageal tumorigenesis and molecular events in rats treated with N-nitrosomethylbenzylamine, which is probably due to the suppression of tumor incidence and multiplicity<sup>[21]</sup>. EGCG is the most abundant and active constituent among tea polyphenols. In general, the anti-carcinogenic activities of EGCG are mediated *via* multiple mechanisms, including the inhibition of mitogen activation protein kinases (MAPK), activator protein-1 and cell transformation<sup>[22-24]</sup>, inhibition of epidermal growth factor receptor (EGFR) phosphorylation<sup>[25]</sup>, induction of cell cycle arrest (G0/G1)<sup>[26,27]</sup> and apoptosis<sup>[28]</sup>, and inhibition of DNA methyltransferase (DNMT) activity<sup>[29]</sup>.

EGCG also regulates multiple targets and mechanisms in protecting against esophageal cancer. EGCG (40  $\mu\text{mol/L}$ ) inhibits phosphorylation of ERK1/2, c-Jun, and cyclooxygenase-2 (COX-2), which are increased in the human esophageal cancer cell lines SKGT-4 and TE-8 as well as in esophageal tissue specimens obtained from patients<sup>[30]</sup>. *In vivo* analysis using nude mouse xenograft models also confirmed that lower tumor formation and growth are associated with the decreased expression levels of phosphorylated extracellular-signal-regulated kinase (ERK) and COX-2 induced by EGCG treatment (50  $\mu\text{g/kg}$  per day)<sup>[30]</sup>. Together these suggest that EGCG may protect against esophageal cancer by reducing pro-inflammatory mediators, including ERK, c-Jun

**Table 1 Chemopreventive phytochemicals and their actions and targets/mechanisms during the development of esophageal cancer, gastric cancer and colon cancer**

	Natural phytochemical	Chemopreventive action	Targets/mechanisms	Ref.
Esophageal cancer	Isothiocyanate	Inhibition of DNA damage	Inhibition of DNA methylation	[17]
		Inhibition of tumorigenesis	Inhibition of cytochrome P450 enzymes activity	[11,18]
	EGCG	Anti-inflammation	Inhibition of phosphorylated ERK1/2, c-Jun and COX-2 expressions	[30]
			Decreased COX-2 expression and PGE <sub>2</sub> production	[32]
		Growth inhibition	Inhibition of EGFR phosphorylation	[31]
		Induction of cell cycle arrest	Inhibition of cyclin D1	[32]
	Curcumin	DNA damage	Inhibition of DNA methyltransferase activity	[29]
		Antioxidant	Induction of SOD-1	[46]
		Anti-inflammation	Inhibition of COX-2	[46]
			Inhibition of NF-κB activity	[50-52]
			Inhibition of IL-8 mRNA expression	[52]
Gastric cancer	Curcumin	Apoptosis and cell cycle arrest	Inhibition of Notch signaling	[57]
		Chemoresistance	Downregulation of NF-κB	[61]
			Induction of apoptotic genes <i>Bcl-2</i> and <i>Bcl-xL</i>	[61]
		Suppression of cell proliferation and invasion	Downregulation of EGFR-PAK1	[62]
	Resveratrol		Reduction of cyclin D1 expression	[62]
		Inhibition of cell cycle progression	Inhibition of PKC	[72,73]
	Sulforaphane		MEK1/2-ERK1/2-c-Jun	[74]
		Induction of apoptosis	Reduction in <i>Bcl-2</i> , enhancing <i>Bax</i> gene	[76,77]
		Protection against oxidative stress	Stimulation of Nrf2	[78-80]
		Anti-bacterial activity	Enhancement of GST and glutathione levels	[78,81]
Colon cancer	Curcumin		Decrease in gastric bacterial colonization	[82]
			Reduction in the expression of TNF-α and IL-1β	[82]
		Growth inhibition	EGFR/IGFR	[102]
			ERK/Egr-1/EGFR	[87,96]
	Resveratrol	Suppression of tumorigenesis	AMPK-COX-2	[101]
			Wnt/β-catenin	[102,106]
		Anti-proliferation Apoptosis	IGF-1, p53	[108]
			PPP-FAK signaling	[109]
		Apoptosis growth inhibition	AMPK	[110]

ERK: Extracellular-signal-regulated kinase; COX-2: Cyclooxygenase-2; PGE<sub>2</sub>: Prostaglandin E<sub>2</sub>; EGFR: Epidermal growth factor receptor; EGCG: Epigallocatechin gallate; SOD: Superoxide dismutase; NF-κB: Nuclear factor κB; IL-8: Interleukin-8; PKC: Protein kinase C; MEK: Mitogen-activated protein kinase; Bcl-2: B-cell lymphoma 2; GST: Glutathione S-transferase; TNF: Tumor necrosis factor; IGFR: Insulin-like growth factor receptor; PPP: Pentose phosphate pathway; FAK: Focal adhesion kinase; AMPK: Adenosine monophosphate-activated protein kinase.

and COX-2 in *in vitro* carcinogenesis and *in vivo* tumorigenesis models, and cancer patients.

EGCG inhibits phosphorylation of EGFR and HER-2/neu in KYSE 150 esophageal squamous cell carcinoma, leading to the inhibition of growth factor receptor and, thus, exerting anti-carcinogenic activity<sup>[31]</sup>. Another study demonstrated that EGCG (4 mg/kg *i.p.*) attenuates cyclin D1 and COX-2 gene expression, thereby reducing the production of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) in rats treated with NMBA<sup>[32]</sup>. This suggests that cyclin D1 and COX-2 may act as partial targets of EGCG. EGCG-mediated decrease in PGE<sub>2</sub> production following NMBA treatment was further supported by another study using F344 rats<sup>[33]</sup>. Lastly, EGCG also inhibits DNA methylation, thereby suppressing the onset of esophageal cancer. Indeed, hypermethylation of DNA is associated with critical events during cancer progression, such as cell cycle regulation, DNA repair, and apoptosis<sup>[34-36]</sup>. In particular, methylation of CpG by DNMT is known to cause chromosome condensation and transcription repression<sup>[37,38]</sup>. The importance of DNA demethylation has been emphasized in strategies to develop cancer therapies and was further supported by studies using

DNMT inhibitors. Treatment with DNMT inhibitors leads to the inhibition of cancer cell growth, induction of cancer cell apoptosis, and attenuation of tumor volume in mice<sup>[39-42]</sup>. In fact, Fang *et al.*<sup>[29]</sup> demonstrated that EGCG acts as a potent DNA methylation inhibitor by suppressing DNMT activity, leading to the demethylation of CpG and reactivation of methylation-silenced genes in the human esophageal cancer cell line KYSE 510.

### Curcumin

Curcumin is a yellow pigment derived from turmeric, the powdered rhizome of *Curcuma longa* Linn. Accumulating evidence suggests numerous health benefits of curcumin, including antioxidant, anti-inflammatory, and anti-carcinogenic properties<sup>[43,44]</sup>, which are probably mediated by regulation of multiple intracellular targets<sup>[5]</sup>. Multiple molecular targets have been identified using various cancer cell lines and xenograft animal models of esophageal cancer.

Curcumin is a well-known antioxidant<sup>[43]</sup>. Excess amounts of reactive oxygen species (ROS) lead to the initiation, progression, and promotion of various cancers<sup>[45]</sup>. Therefore, the role of antioxidants is critical dur-

ing the development of cancers. In fact, treatment with curcumin (10-100  $\mu\text{mol/L}$ ) reversed suppression of the powerful antioxidant superoxide dismutase (SOD)-1 and induction of COX-2 gene expression following treatment with bile acid in an esophageal epithelial cell line (HET-1A)<sup>[46]</sup>. This suggests that the antioxidant capacity of curcumin contributes to the prevention of esophageal cancer by increasing the activity and/or expression levels of antioxidant enzymes, including SOD, and reducing pro-oxidant enzymes, such as COX-2.

In addition to its antioxidant capacity, curcumin exerts its anti-cancer activities *via* its anti-inflammatory activity. Nuclear factor  $\kappa\text{B}$  (NF- $\kappa\text{B}$ ) is a well-known pro-inflammatory transcription factor involved in the initiation, promotion and progression of cancers<sup>[47]</sup>. It is also known that increased NF- $\kappa\text{B}$  activity is associated with greater cell proliferation, invasion, angiogenesis, metastasis, suppression of apoptosis, and chemoresistance in various types of cancer<sup>[48,49]</sup>. Several studies have demonstrated that curcumin inhibits NF- $\kappa\text{B}$  activity in esophageal adenocarcinoma<sup>[50,51]</sup>. Rawat *et al.*<sup>[52]</sup> reported that curcumin (50  $\mu\text{mol/L}$ ) protects against bile acid-induced enhanced NF- $\kappa\text{B}$  activity in an esophageal cell line (OE33), which in turn reduces the expression levels of NF- $\kappa\text{B}$  target genes, including interleukin (IL)-8. In addition, patients with Barrett's esophagus supplemented with curcumin (a 500 mg curcumin tablet daily for 7 d) showed decreased IL-8 mRNA expression, suggesting that curcumin can act as a potential chemopreventive agent against esophageal cancer<sup>[52]</sup>.

Recently, it was demonstrated that curcumin induces cell death (apoptosis) and cell cycle arrest by blocking Notch signaling pathways. Notch signaling was recently found to be upregulated in esophageal cancer and is as a therapeutic target for esophageal cancer due to its critical roles in tumor cell proliferation, apoptosis and stem cell maintenance and renewal<sup>[53-56]</sup>. Inhibition of Notch signaling in oral squamous carcinoma cells by curcumin also contributes to downregulation of NF- $\kappa\text{B}$ , which in turn reduces the expression of target genes of NF- $\kappa\text{B}$ , including Bcl-2, cyclin D1, vascular endothelial growth factor, and matrix metalloproteinase-9<sup>[57]</sup>.

## PHYTOCHEMICALS IN GASTRIC CANCER

Gastric cancer is the seventh most common cause of cancer-related mortality in the world. Exposure to chemical carcinogens or *Helicobacter pylori* (*H. pylori*) infection causes several events which may lead to the development of gastric cancer<sup>[58]</sup>. In particular, *H. pylori* infection results in infiltration of neutrophils and macrophages into the gastric mucosa. Infiltration of neutrophils and macrophages leads to the production of free radicals, including superoxide and nitric oxide. ROS-mediated stress responses result in gastric mucosal injury, ulcers, and ultimately gastric cancer<sup>[59]</sup>. Therefore, agents that have powerful antioxidant potential *via* scavenging ROS or enhancing antioxidant capacity may help to protect against gastric cancer development (Table 1).

### Curcumin

It has been suggested that curcumin inhibits *H. pylori* infection in mice by reducing its growth<sup>[60]</sup>. The mechanisms of cellular growth and potential therapeutic capacity of curcumin have been further investigated by *in vitro* studies using multiple gastric cancer cell lines. Curcumin protects against chemoresistance in human gastric cancer cells by downregulating NF- $\kappa\text{B}$  and subsequent NF- $\kappa\text{B}$ -mediated anti-apoptotic genes, such as Bcl-2 and Bcl-xL in the human gastric cancer SGC 7901 cell line<sup>[61]</sup>.

In addition, curcumin reduces EGFR expression and the activity of p21-activated kinase (PAK)1, a downstream regulator of EGFR. Curcumin also reduces NF- $\kappa\text{B}$  activity, regulated by PAK1, leading to decreases in cell proliferation by reducing the mRNA and protein expression of cyclin D1 and suppresses cell cycle progression from the G1 to S phases. Therefore, curcumin inhibits the proliferation and invasion of various gastric cancer cells<sup>[62]</sup>.

### Resveratrol

Resveratrol is a highly abundant polyphenol found in red grapes and red wine. Epidemiological studies have revealed an inverse relationship between red wine consumption and the incidence of cardiovascular disease<sup>[63,64]</sup>. The cardioprotective effect of red wine was attributed to resveratrol<sup>[65]</sup>. During the last two decades, extensive research has focused on the antioxidant, anti-inflammatory and anti-carcinogenic health benefits of resveratrol<sup>[66]</sup>. Specifically, resveratrol was found to have antibacterial effects<sup>[67]</sup> by inhibiting the growth of multiple *H. pylori* strains<sup>[68-70]</sup>. Increased expression of IL-8 and increased production of ROS were detected in the gastric mucosa following exposure to *H. pylori*. Furthermore, *H. pylori*-mediated infection increases motility and leads to morphological changes in co-cultured cells, known as the hummingbird phenomenon. Treatment with resveratrol (1-100  $\mu\text{mol/L}$ ) significantly attenuated IL-8 secretion, ROS formation, and markedly inhibited morphological changes in cells infected with *H. pylori*<sup>[71]</sup>. Hence, resveratrol is a candidate therapeutic agent against gastric cancer.

Resveratrol inhibits cell cycle progression of nitrosamine-stimulated KATO-III and RF-1 cells by inducing cell cycle arrest in the G0/G1 phase through inhibiting kinase C-mediated mechanisms and induces apoptotic cell death in various gastric adenocarcinoma cell lines<sup>[72,73]</sup>. Another mechanism by which resveratrol regulates cell proliferation is associated with the MEK1/2-ERK1/2-c-Jun signaling cascade, a critical signaling pathway in the proliferation and growth of human adenocarcinoma gastric cells. Resveratrol was found to suppress the phosphorylation of MEK1/2-ERK1/2, which subsequently inhibits translocation of c-Jun into the nuclear compartment, leading to inhibition of cell proliferation<sup>[74]</sup>.

In addition to the inhibition of cell proliferation, resveratrol (50-200  $\mu\text{mol/L}$ ) induces apoptosis in human gastric cancer SGC7901 cells by producing ROS, which can be reversed by treatment of cells with SOD or catalase, leading to the attenuation of resveratrol-mediated



cellular apoptosis<sup>[75]</sup>. Resveratrol induces apoptosis in esophageal carcinoma (EC-9706) cells, mediated by reducing the expression of Bcl-2 and enhancing that of the pro-apoptotic gene Bax<sup>[76]</sup>. Resveratrol can induce apoptosis of transplanted tumor cells, probably mediated by downregulation of the anti-apoptotic gene bcl-2 and upregulation of the apoptotic gene Bax by resveratrol in implanted primary human gastric cancer cells in nude mice<sup>[77]</sup>.

### Sulforaphane

The natural chemical compound sulforaphane is an isothiocyanate and is abundant in cruciferous vegetables, especially broccoli<sup>[59]</sup>. Sulforaphane is present as sulforaphane glucosinolates (SGS), which is biologically inactive. SGS is hydrolyzed by the action of myrosinase in the oral cavity and small intestine to produce sulforaphane. Biologically active sulforaphane is ultimately absorbed into the systemic circulation, where it exerts various activities<sup>[59]</sup>. Although sulforaphane is not itself an antioxidant, it exerts antioxidant activity by stimulating Nrf2-dependent antioxidant enzymes, such as glutathione S-transferase (GST), thereby protecting cells against oxidative stress<sup>[78-80]</sup>. Compared to other strong antioxidants, such as vitamin C or polyphenols, sulforaphane maintains the activities of antioxidant enzymes, including NAD(P)H:quinone oxidoreductase (NQO1) and GST in the gastric mucosa of Nrf2<sup>-/-</sup> mice infected with *H. pylori* and fed a high salt diet<sup>[59]</sup>. This renders sulforaphane a more potent antioxidant substance and mediates its protection of the gastric mucosa against oxidative stress.

Sulforaphane increases detoxification as well as antioxidant enzymes in a Nrf2-dependent manner. Fahey *et al.*<sup>[78]</sup> demonstrated that sulforaphane suppresses benzo[a]pyrene-evoked forestomach tumors in ICR mice. This is probably mediated by inducing phase 2 detoxification enzymes, including NQO1 and GST, and upregulating antioxidant enzymes, which are abrogated in mice without the Nrf2 gene<sup>[78]</sup>. In patients with *H. pylori*-associated gastritis, *H. pylori* eradication increased or restored the activity of GST and glutathione levels in the antral mucosa<sup>[81]</sup>. This further emphasizes the importance of antioxidants during the development of gastric cancers associated with *H. pylori* infection.

In addition to its antioxidant capacity, sulforaphane exerts chemoprotective effects which are attributed to its *in vitro* anti-bacterial activity<sup>[59]</sup>. In a clinical study of *H. pylori*-infected patients ( $n = 48$ ), the group that consumed broccoli (70 g/d; containing 420  $\mu$ mol/L sulforaphane precursor) for 8 wk showed decreased levels of markers of *H. pylori* colonization (*i.e.*, urease level and *H. pylori* stool antigen) and markers of gastric inflammation (*i.e.*, serum pepsinogens I and II) compared to the placebo group<sup>[82]</sup>. An *in vivo* study using C57BL/6 female mice infected with *H. pylori* Sydney strain 1 and maintained on a high-salt (7.5% NaCl) diet confirmed the anti-bacterial activity of sulforaphane. Mice treated with broccoli rich in sulforaphane showed decreased gastric bacterial colo-

nization as well as reduced expression of tumor necrosis factor (TNF)- $\alpha$  and IL-1 $\beta$  in the gastric mucosa, contributing to amelioration of inflammation and, thus, prevention of high salt-induced gastric corpus atrophy<sup>[82]</sup>. Interestingly, the anti-bacterial and anti-inflammatory activities of sulforaphane were not observed in mice with Nrf2 gene depletion, suggesting that sulforaphane exerts its effect *via* Nrf2<sup>[82]</sup>.

## PHYTOCHEMICALS IN COLORECTAL CANCER

Colorectal cancer is one of the most commonly diagnosed cancers in both males and females<sup>[83]</sup>. The mortality rate of males with colon and rectal cancer was the third highest for cancers in the United States between 1930 and 2007<sup>[84]</sup>. Consumption of a high-calorie diet that is high in fat leads to obesity. Many studies have investigated the contribution of obesity to colorectal diseases<sup>[85-88]</sup>. The colon is one of the first organs to encounter various factors in foods and, thus, the effects of natural bioactive compounds in the diet on colorectal tissue are the subject of extensive investigation.

The Wnt signaling pathway is a primary factor in colorectal cancer. Among several Wnt signaling proteins,  $\beta$ -catenin is a key regulator, which turns on and off cell proliferation proteins. In the normal state, the “destruction” complex comprising axin, APC, and glycogen synthase kinase-3 $\beta$ , phosphorylates  $\beta$ -catenin, which subsequently becomes degraded<sup>[89]</sup>. However, after activation of the Wnt signaling pathway, the “destruction” complex is suppressed and  $\beta$ -catenin is not degraded by ubiquitination. Accumulated  $\beta$ -catenin then translocates into the nucleus and binds directly to the T-cell factor (TCF)/lymphoid enhancer factor (LEF) family molecules. These interactions stimulate TCF/LEF target genes involved in cellular proliferation, such as c-myc and cyclin D1<sup>[90,91]</sup>.

More importantly, the Wnt signaling pathway is stimulated by obesity<sup>[92]</sup> and is a secondary factor in the development of colon cancers<sup>[93,94]</sup>. Obesity is associated with chronic inflammation<sup>[85]</sup>. In obesity-related cancers, phosphoinositide-3-kinase (PI3K)/Akt, MAPK, and their downstream signaling proteins, including mammalian target of rapamycin (mTOR), are activated as the severity of obesity increases<sup>[95]</sup>. Overall, it is commonly accepted that suppression of the inflammatory signaling pathway may represent an important strategy for inhibition of both Wnt- and obesity-related colon cancers. Several lines of evidence have reported the anti-colon carcinogenic effects of natural compounds, which act as small molecule inhibitors of the inflammatory signaling pathway. Among them, curcumin and resveratrol are the most significant anti-carcinogenic compounds (Table 1).

### Curcumin

Curcumin is the yellow pigment of turmeric and numerous studies using various carcinogenesis models have



shown its chemopreventive effects. One clinical study reported that curcumin has anti-colon carcinogenic effects. Indeed, oral intake of curcumin (4 g for 30 d) decreased the number of aberrant crypt foci in the colon in this Phase IIa clinical trial of curcumin for the prevention of colorectal neoplasia.

A number of studies have investigated the mechanisms underlying the inhibition of colon cancer development by curcumin. The major targets of the signaling pathways regulated by curcumin are EGFR<sup>[96,97]</sup>, AMPK-COX-2<sup>[98]</sup>, MAPK<sup>[99]</sup> and Wnt/ $\beta$ -catenin<sup>[100]</sup>. EGFR is one of four family erbB receptors and is involved in many malignancies, including colorectal cancer<sup>[97]</sup>, by modulating multiple signaling pathways. Specifically, ligand-activated EGFRs are autophosphorylated and activate Ras and other signaling pathways, which in turn increase the expression of EGFR target genes. Indeed, increased levels and function of EGFR are closely associated with the metastatic potential of human colon carcinoma cells. Chen *et al.*<sup>[96]</sup> reported that curcumin inhibits colon cancer cell growth by reducing the ERK/Egr-1/EGFR signaling pathway and decreasing the expression of EGFR. Curcumin was also demonstrated to prevent the emergence of chemoresistant colon cancer cells *via* inhibition of EGFR and insulin-like growth factor (IGF)-1R.

AMPK is a highly conserved kinase in eukaryotes. Although its main function is in maintenance of energy homeostasis, novel roles for AMPK were discovered recently. The AMPK-COX-2 cascade is an important pathway associated with cancer growth. Previous studies demonstrated that a signaling cascade involving AMPK, pAkt and COX-2 is a promising target as it is regulated by curcumin during cancer cell growth. Over 80% of colonic adenomas and carcinomas exhibit mutations in the APC gene and constitutive activation of Wnt signaling<sup>[101]</sup>. Thus, Wnt/ $\beta$ -catenin signaling has been targeted for the development of novel anti-colorectal cancer drugs. Curcumin also inhibits the Wnt/ $\beta$ -catenin signaling pathway<sup>[102]</sup>. Curcumin impairs Wnt signaling and cell-cell adhesion pathways, subsequently inducing G<sub>2</sub>/M phase and apoptosis in colon cancer cells.

### Resveratrol

Resveratrol is a naturally occurring phenolic phytochemical, which is present in red grapes. Resveratrol exerts its chemopreventive and chemotherapeutic effects by modulating multiple biological activities. Daily *p.o.* doses of 0.5 g or 10 g resveratrol for 8 d inhibited tumor cell proliferation by 5% with no resveratrol-related adverse effects in patients with resectable colorectal cancer ( $n = 90$ )<sup>[103]</sup>. Another clinical study demonstrated that ingestion of grape powder suppresses the expression of Wnt target genes, including cyclin D1 and axin II, in normal colonic mucosa. This suggests that dietary supplementation with resveratrol-containing products is a potential colon cancer preventive strategy and that Wnt/ $\beta$ -catenin is a potential target for resveratrol in normal colonic mucosa<sup>[104]</sup>. The anti-colonic tumor effects of resveratrol have been investigated in various *in vivo* studies<sup>[105-107]</sup>.

Resveratrol (300 ppm) supplementation reduced levels of markers of DSS-mediated colitis inflammation, such as iNOS, COX-2 and TNF- $\alpha$ , in mice<sup>[105]</sup>. Resveratrol also inhibited the 1,2-dimethylhydrazine-induced tumor burden per animal, per group and over the three regimens of colon carcinogenesis (initiation, post-initiation and entire period)<sup>[107]</sup>.

Resveratrol suppresses IGF-1-induced human colon cancer cell proliferation by activating p53 signaling pathways. Because the IGF signaling pathway is closely related to obesity-mediated colorectal carcinogenesis<sup>[108]</sup>, resveratrol may be useful for suppressing obesity-induced colorectal cancers. Additionally, resveratrol inhibits human colon cancer cell proliferation and promotes apoptosis by suppressing the pentose phosphate pathway and focal adhesion kinase, a critical protein for cell-extracellular matrix communication. This supports the anti-colon-carcinogenic effect of resveratrol in obese individuals<sup>[109]</sup>.

Furthermore, resveratrol exerts synergistic anti-cancer effects on chemoresistant cancer cells by regulating the AMPK signaling pathway. The HT-29 cell line has been used to develop anti-cancer drugs intended to overcome chemoresistance<sup>[110]</sup>. Although 100  $\mu$ mol/L etoposide, an anti-cancer agent, did not inhibit the proliferation of HT-29 cells, pretreatment with resveratrol (50-400  $\mu$ mol/L) induced cytotoxicity under 100  $\mu$ mol/L etoposide<sup>[110]</sup>. Additionally, the phosphorylation level of acetyl-CoA carboxylase (ACC), the downstream molecule of AMPK, was increased by co-treatment with resveratrol and etoposide and increased phospho-ACC and pAMPK inhibition of cell viability. Compound C, an AMPK inhibitor, reduced resveratrol/etoposide-induced cytotoxicity on HT-29 cells<sup>[110]</sup>.

## CONCLUSION

Gastroenterological cancers, including those of the esophagus, stomach and colon, are closely associated with lifestyle factors, especially diet. Patients suffering from gastroenterological cancers often cannot be completely cured with regular chemopreventive strategies and, thus, prevention, suppression, and/or delaying the onset of these cancers are critical. A number of natural phytochemicals, including curcumin, resveratrol, isothiocyanates, EGCG, and sulforaphane have been shown to have anti-carcinogenic, anti-inflammatory, and antioxidant activities by targeting small molecules or regulating signaling cascades, thereby protecting against the development of gastroenterological cancers. Although most phytochemicals act as small molecule inhibitors, they often have low bioavailability following oral administration. Indeed, the concentration of resveratrol is lower than its major metabolite resveratrol sulfate glucuronide after daily administration of 0.5 g resveratrol for 8 d to colorectal cancer patients<sup>[103]</sup>. The majority of the phytochemicals are readily converted to their metabolites in the gastrointestinal tract. These metabolites may have similar or better effects than their parent compounds and, thus, may also represent primary therapeutic agents.

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## Nutritional modulators of ulcerative colitis: Clinical efficacies and mechanistic view

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ance have been suggested as having important roles in inducing changes in the microbial population and intestinal barrier integrity and in regulating inflammatory immune responses, directly or indirectly. Excess energy intake is now known to increase pathogenic microbial populations. Likewise, the application of appropriate probiotics may reverse the pathogenic progression of the disease. In the meantime, dietary anti-inflammatory compounds, including omega-3 fatty acids and other phytochemicals, may directly suppress inflammatory responses in the course of UC development. In this review, the increased prevalence of UC and its management are interpreted from the standpoint of nutritional modulation to regulate the intestinal microflora population, intestinal epithelium permeability, and inflammatory responses.

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### Abstract

Ulcerative colitis (UC) is an inflammation-associated disease of the colon and rectum. The onset and progress of the disease are directly influenced by the nature of the intestinal microflora, the intestinal barrier function, and the immunological responses of the host. The epithelial invasion of pathogenic bacteria due to excess contact and/or barrier dysfunction is related to inflammation mediated by intestinal immune responses. Although the etiology of UC is not clearly understood, recent studies have shown a rising incidence of UC worldwide, and this phenomenon is more prominent in Asian countries and in Asian immigrants in Western countries. The increased prevalence of UC also contributes to an increased risk of developing colorectal cancer. Environmental factors, including changes in dietary habits, have been suggested as major risk factors of UC. A systematic review showed a negative association between UC risk and vegetable intake, whereas total fat, omega-6 fatty acids and meat intake were positively associated with an increased risk of UC. Individual dietary factors and energy bal-

**Key words:** Ulcerative colitis; Intestinal microflora; Immunity; Inflammation; Clinical; Obesity; Probiotics; Omega-3 fatty acids; Antioxidants

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### INTRODUCTION

Ulcerative colitis (UC) is a major type of inflammatory bowel disease (IBD) characterized by chronic inflammation in the colon and rectum. It progresses by extensive epithelial apoptosis and ulceration due to chronic inflammation induced by T-helper (Th) 2 cytokines<sup>[1]</sup>. Recent studies have also indicated that a balance between proinflammatory Th17 cells and immunosuppressive Treg cells play a crucial role in the development of

UC<sup>[2]</sup>. Although the etiology of UC has not been clearly determined, environmental factors are thought to stimulate overt immune responses to bacterial components in individuals with high genetic susceptibility. A recent report on the incidence of IBD in Asia indicated that the prevalence of UC is growing rapidly in Japan, Hong Kong, and South Korea<sup>[3,5]</sup>, countries where IBD used to be rare. The most recent reports on UC prevalence in Japan and South Korea provided figures of 63.6 and 30.9 per 100 000 people, respectively<sup>[4,5]</sup>. UC reportedly affects 0.24% of the United States population<sup>[6]</sup>, and the prevalence in Northern European countries ranges from 40 to 240 per 100 000 people<sup>[7]</sup>.

Lifestyle changes, as well as increased awareness of the disease and improved diagnosis, may have contributed to the increased incidence. Dietary habits in Asian countries have changed, resulting in a Western-style diet with fewer plant-based and more processed foods. A recent systematic review of 19 studies reported a negative association between UC risk and vegetable intake, whereas total fat, omega-6 fatty acids, and meat intake were positively associated with increased UC risk<sup>[8]</sup>. Information is limited, however, on the role of individual dietary components in UC development, and most nutritional modulation studies have focused on delaying relapses of UC, efforts that involve secondary rather than primary prevention. The cumulative incidence of relapse in UC are 30%, 72%, and 88% after 1, 5, and 10 years following the initial diagnosis<sup>[9]</sup>. Patients younger than 40 years have been shown to present with more severe disease at the time of diagnosis compared with older patients<sup>[10]</sup>. Importantly, the increased incidence of UC may be closely related to an increase in the prevalence of colorectal cancer (CRC). A subset study population with UC from the Kaiser Permanente Medical Care Program was analyzed for CRC incidence and mortality, and the standardized mortality ratio for CRC among UC patients was 2.0 (95%CI 1.3-2.7)<sup>[11]</sup>. Chronic inflammation mediates a wide range of signaling cascades that possibly facilitate colorectal carcinogenesis, and UC remission may reduce the risk of CRC. In this review, the pathophysiology of UC is summarized to allow understanding of the molecular mechanisms involved in the action of dietary components. Major dietary components reported to regulate, directly or indirectly, inflammatory responses in UC are discussed, with a focus on human studies where available. Genetic factors and therapeutic measures, while important to consider, fall outside the scope of this review.

## INTESTINAL BARRIER FUNCTIONS AND INFLAMMATION IN UC

The human large intestine contains a concentration of approximately  $10^{11}$ - $10^{12}$  microorganisms per gram of luminal content. These microbes can be either beneficial or harmful to the intestinal epithelium. Under normal circumstances, multiple mechanisms protect the intestinal epithelium from microbial invasion, but environ-

mental stimuli in combination with genetic factors can facilitate overgrowth of harmful microflora and induce abnormal immune responses that disrupt the mucosal barrier, causing inflammation (Figure 1).

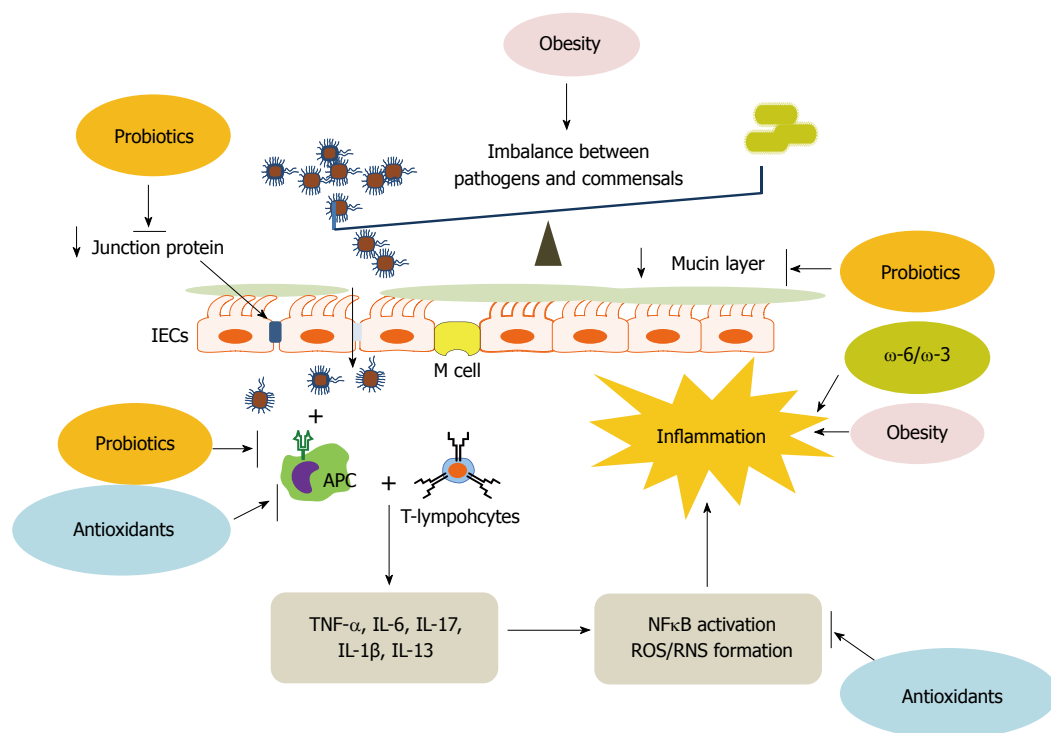
The luminal side of the intestinal membrane is in constant contact with intestinal microflora. The most evident characteristic of pathogenic bacteria are their invasiveness and induction of inflammatory responses in the intestinal epithelium<sup>[12]</sup>. The host system, in other words, is able to recognize and differentiate pathogens from the commensals. To protect the host from pathogenic bacteria, the intestinal epithelium is equipped with multiple defense systems. Intestinal epithelial cells (IECs), which are layered by glycoproteins such as mucin, form the first line of immune defense. Tight junction (TJ) proteins seal the space between IECs. The layer below the IECs is the sub-epithelial dome, containing antigen-presenting cells, and underneath are Payer's patches, where B-cell follicles and T-cells reside.

Bacterial invasion is recognized by receptors called pattern recognition receptors (PRRs) in the IEC membrane, and the primary PRRs are toll-like receptors (TLRs). There are 10 different classes of TLRs expressed throughout the whole human gastrointestinal tract. The recognition of bacteria is carried out by means of communication between PRRs and microbial components such as lipopolysaccharide (LPS), and this is followed by immune responses to destroy the invading pathogens. Microbiota and viral-associated ligands use different types of TLRs depending on molecular patterns. For example, TLR2 recognizes lipopeptides, TLR 3 recognizes viral-derived dsRNA, and TLR4 recognizes LPSs<sup>[13]</sup>. Constant immune responses provoke chronic inflammation, which is mediated by pro-inflammatory cytokines and chemokines. The inflammation not only contributes to clinical features of inflammatory disease, but also exacerbates the penetration of pathogenic bacteria by increasing the membrane permeability, creating a vicious cycle. The ability of the intestinal epithelium to distinguish commensal from pathogenic bacteria is important because the intestine requires a symbiotic relationship with commensals without immune responses. Several explanations exist for the intestinal epithelium's ability to discriminate harmful bacteria from commensals<sup>[14]</sup>. In brief, pathogenic bacteria possesses virulence factors to stimulate the innate immune responses while commensals mutate their molecular patterns, escaping recognition by TLRs, and attenuate the nuclear factor (NF)- $\kappa$ B pathway. Also, the hyporesponsiveness to commensals has been explained by the anti-inflammatory nature of the gut mucosa including reduced expression of PRRs and reduced inflammatory nature of intestinal immune cells.

## DIETARY FACTORS MODULATING INTESTINAL BARRIER FUNCTIONS IN UC

### *Excess energy (obesity)*

Obesity is the most convincing risk factor in the devel-



**Figure 1** Disrupted intestinal homeostasis in ulcerative colitis and the role of nutritional factors. Ulcerative colitis is a chronic inflammatory disease of the colon and rectum. Inflammatory responses are induced by the penetration of excessive pathogenic bacteria due to the increased population of pathogenic bacteria, the loss of junction proteins and thin mucin layer. Once pathogens are recognized by antigen-presenting cells (APC), T-lymphocytes produce pro-inflammatory cytokines activating inflammation-inducing nuclear transcription factor, nuclear factor (NF)- $\kappa$ B and generating reactive oxygen species (ROS) and reactive nitrogen species (RNS) which result in the inflamed intestine. Obesity is known to cause imbalances between pathogens and commensals as well as chronic inflammation. The increased  $\omega$ -6 to  $\omega$ -3 fatty acid ratio in the diet also accelerates inflammatory responses. Probiotics supplementation helps to maintain gut health by retaining tight junctions and mucin layer. Probiotics and antioxidants suppress immoderate immune responses, and ROS-induced inflammatory responses are moderated by antioxidants. TNF- $\alpha$ : Tumor necrosis factor- $\alpha$ ; IL-1 $\beta$ : Interleukin-1 $\beta$ ; IECs: Intestinal epithelial cells.

opment of many noncommunicable diseases, including type 2 diabetes, cardiovascular diseases, non-alcoholic fatty liver disease, and selected types of cancer. World Health Organization statistics stated that physical inactivity and being overweight or obese contributed, respectively, to 5% and 6% of deaths<sup>[15]</sup>. The estimated prevalence of overweight [body mass index (BMI)  $\geq 25$  kg/m<sup>2</sup>] and obese (BMI  $\geq 30$  kg/m<sup>2</sup>) males and females aged 15 or older was  $\geq 80\%$  in the United States,  $\geq 65\%$  in Canada, Mexico, Argentina, Australia, the United Kingdom, and Germany, and  $\geq 35\%$  in all other countries, except for several African countries, India, and South East Asian countries<sup>[16]</sup>.

Obesity and abdominal obesity have been specifically identified as the most convincing risk factors for the development of CRC<sup>[17]</sup>. In many studies, obesity-related inflammatory biomarkers were shown to be associated with disease activity. Among adipokines, resistin was considered to have a positive relationship with disease activity<sup>[18,19]</sup>. In a multicenter registry of children with IBD, 1 in 3 children with UC were overweight or obese, which is comparable to the rate in the general population<sup>[20]</sup>. However, prior IBD-related surgery was positively associated with being overweight or obese, which implies that obesity may provoke a more severe disease course. CRP was found to be a marker predictive of disease progression in a population-based prospective

study<sup>[21]</sup>. CRP concentrations above 23 mg/L at diagnosis showed an odds ratio (OR) of 4.8 (95%CI, 1.5-15.1) in the prediction of surgery in UC patients. These results suggest that systemic inflammation worsens disease activity, and therefore the suppression of inflammatory events may be critical for the management of UC.

Mechanistic explanations have not been provided regarding the relationship between obesity and intestinal inflammation in patients. However, a number of rodent model studies have indicated that intestinal inflammation is mediated through obesity-related factors. Leptin was shown to act as a critical mediator in colitis development<sup>[22]</sup>. Leptin-deficient (*ob/ob*) mice were shown to be resistant to chemically-induced colitis, indicating that leptin is a critical regulator. Adiponectin, on the other hand, was shown to be a negative regulator in the development of dextran sulfate sodium (DSS)-induced colitis through the suppression of pro-inflammatory cytokine and chemokine production<sup>[23]</sup>. In a recent study, the effects of high-fat diet-induced obesity and chemically-induced colitis were compared in the development of UC<sup>[24]</sup>. A high-fat diet alone did not induce characteristic histopathological features of UC, indicating UC development is accelerated through inflammatory infiltration in the colon tissue of chemically-induced colitis. It is also interesting to note that the inflamed intestines of mice with chemically-induced colitis exhibited higher

inflammatory activities in the mesenteric fat compared with obese mice, which implies that intestinal inflammation may precede systemic inflammation<sup>[25]</sup>. These results suggest that obesity may not be an independent risk factor for UC development, although it may aggravate disease progression.

There are several reports showing evidence of obesity-associated changes in the intestinal microbiota. However, there is no clear explanation if this is due to adiposity or dietary composition<sup>[26]</sup>. Therefore, the altered microbial population is either a cause or a consequences of obesity. A recent report suggested that diets high in saturated fat promote taurine-conjugated bile acid formation, altering microbial composition towards the overgrowth of a sulfite-reducing pathobiont, *Bilophilla wadsworthia*. This accelerated the development of colitis in interleukin (IL)-10  $-/-$  mice<sup>[27]</sup>, providing evidence of a direct contribution from increased dietary fat. High-saturated fat diets altered microbial composition, resulting in a significantly higher ratio of *Firmicutes* to *Bacteroidetes*<sup>[28]</sup>. A decreased proportion of *Bacteroidetes*<sup>[29]</sup> was also observed in IBD, suggesting a possible mediating effect of these bacteria in obesity-related pathogenic changes in the intestinal epithelium. Others have reported that changes in the gut microbiota directly contribute to the development of obesity and related metabolic disturbances<sup>[30,31]</sup>, which requires further investigation.

### Fatty acids

The intakes of pro-inflammatory omega-6 fatty acids and anti-inflammatory omega-3 fatty acids have been suggested as important regulators in UC disease activity. A recent systematic review concluded that available randomized controlled trials do not indicate that omega-3 fatty acids are useful to alleviate IBD<sup>[32]</sup>.

Epidemiological studies have been conducted to investigate the association between UC and nutrients. A total of 139 UC patients were identified as a subgroup of the population participating in a large prospective cohort study, the European Prospective Investigation into Cancer and Nutrition (EPIC)<sup>[33]</sup>. Results indicated that there is a marginally significant association between UC and an increasing percentage intake of energy from total polyunsaturated fatty acids (OR, 1.19; 95%CI, 0.99-1.14,  $P = 0.07$ ). In another EPIC sub-cohort report, the highest quartile of intake of linoleic acid was positively associated with UC risk (OR, 2.49; 95%CI, 1.23-5.07)<sup>[34]</sup>. A nested United Kingdom cohort study analyzed the effect of total and specific dietary omega-3 fatty acids intake on the risk of UC<sup>[35]</sup>. Docosahexaenoic acid (DHA) was found to have a statistically significant protective OR of 0.43 (95%CI, 0.22-0.86), while total omega-3 fatty acids and eicosapentaenoic acid (EPA) showed marginally negative values.

Few studies have reported fatty acid composition markers of UC in human biospecimens. In accordance with the above mentioned epidemiological evidence, one study showed that the erythrocyte membrane content of linoleic acid, the most abundant dietary polyunsaturated

acid present in plant seed oil was significantly higher in IBD patients (29 UC and 20 CD patients) compared with control subjects ( $n = 31$ )<sup>[36]</sup>. In the same study, UC patients showed a significantly higher ratio of arachidonic acid (AA) to EPA compared with control subjects. Leukotrienes (LTs) are pro-inflammatory lipid mediators involved in the progression of UC. The 5-lipoxygenase pathway (5-LOX) catalyzes the formation of LTs from AA, and the deletion of the gene encoding 5-LOX in an animal model prevented the development of chemically-induced colitis<sup>[37]</sup>. In a prospective case-control study, UC patients had significantly higher urinary LTE4 excretion compared with controls<sup>[38]</sup>. A subpopulation of the EPIC prospective cohort study showed that subjects in the highest quartile for adipose tissue AA concentration had a relative risk for UC of 4.16 (95%CI, 1.38-2.27)<sup>[39]</sup>.

Experimental animal model studies have investigated the effects on UC development of omega-3 fatty acid supplementation or an increased ratio of omega-3 to omega-6 fatty acid in the diet<sup>[41-43]</sup>. Possible mechanistic explanations include: (1) the restoration of membrane TJ protein expression and distribution; or (2) the activation of peroxisome proliferator-activated receptor- $\gamma$ , possibly inhibiting NF $\kappa$ B transcriptional activity. However, Varnalidis *et al*<sup>[42]</sup> suggested that the ameliorating effects of omega-3 fatty acids are accompanied by increased colonic neutrophil infiltration, and needs further clarification. Matsunaga *et al*<sup>[41]</sup> reported that feeding fish oil (8% w/w) exacerbates DSS-induced colitis in mice by suppressing the expression of adiponectin in myofibroblasts of the intestinal epithelium.

Apart from omega-6 and omega-3 fatty acids, short chain fatty acids have been suggested to suppress intestinal inflammation through G-protein-coupled receptor 43 (GPR-43)<sup>[44]</sup>. Acetate supplementation suppressed DSS-induced colonic inflammation in wild-type mice, while GPR-43  $-/-$  mice did not respond to acetate. These results explain the role that intestinal microbiota play in producing short chain fatty acids from dietary fiber in the maintenance of a healthy gut.

The efficacy and safety of fish oil in the remission of IBD [Crohn's disease (CD) or UC] have been investigated in clinical trials. A recent meta-analysis retrieved 9 randomized, placebo-controlled trial of fish oil administered for at least 6 mo<sup>[45]</sup>. A total of 1.8-6.2 g/d of DHA + EPA or total omega-3 fatty acids were provided during the study period, and the primary outcome was relapse rate. A meta-analysis indicated that there was no significant reduction in the relative risk of UC, while the pooled relative risk of CD relapse was 0.77 (95%CI, 0.61-0.98). A clinical trial based on n-3 polyunsaturated diet therapy (n-6 to n-3 fatty acid ratio of approximately 1) reported that subjects exhibiting disease remission after a n-3 PUFA diet had a higher ratio of n-3/n-6 fatty acids in the red cell membrane compared with the relapse group<sup>[46]</sup>.

### Probiotics

Probiotics are live, nonpathogenic microorganisms that



contribute to the improvement of epithelial and mucosal barrier function through various mechanisms, including reduced intestinal pH, inhibition of pathogenic bacteria, and modulation of the intestinal mucosa immune responses<sup>[47-49]</sup>. Although there is a growing body of evidence from *in vivo* studies on the beneficial effects of probiotics in UC, reliable clinical trials are limited in number. The aim of this chapter is to review randomized, double-blind, placebo-controlled clinical trials which suggest that probiotics are effective in UC.

The probiotic *Escherichia Coli* strain Nissle 1917 (EcN) is non-pathogenic and one of the best characterized strains used as a probiotic drug<sup>[50]</sup>. The clinical effectiveness of EcN in adult UC patients seems to be promising<sup>[51]</sup>. Patients with moderate distal active UC were assigned to treatment with either 10, 20, or 40 mL of EcN enema ( $n = 23, 23, 24$ ) containing  $10^8$  CFU/mL or placebo once a day for at least 2 wk. If there was no disease activity index (DAI) improvement after 2 wk, patients were classified as non-responders and discontinued therapy. Those classified as responders could receive the treatment for 4 or 8 wk. Although the number of responders was not significantly higher in the EcN group than in the placebo group, the efficacy of rectal EcN treatment was dose-dependent and significant in the per-protocol analysis. This Phase II study showed that EcN is an effective and well-tolerated alternative or supplementary treatment in mild-to-moderate distal UC patients.

The induction of remission in patients with mild-to-moderate UC was also demonstrated with VSL3™ treatment. VSL3 is a high-concentration probiotic mixture, which combines 8 different bacterial species including *Bifidobacterium* (*B.*) *longum*, *B. breve*, *B. infantis*, *Lactobacillus* (*L.*) *casei*, *L. plantarum*, *L. acidophilus*, *L. Bulgaricus*, and *Streptococcus thermophilus*<sup>[52]</sup>. In a multicenter, randomized, double-blind, placebo-controlled trial, subjects received either VSL3 ( $n = 77$ ) containing  $3.6 \times 10^{12}$  CFU or placebo ( $n = 70$ ) twice per day for 12 wk<sup>[53]</sup>. VSL3 led to a 50% improvement in DAI at 6 wk and clinical remission was shown in 33 patients given VSL3 (42.9%) compared with 11 patients given placebo (15.7%) at 12 wk.

A single-center, randomized, double-blind, placebo-controlled trial of the efficacy of symbiotic treatment was performed using 18 patients with active UC<sup>[54]</sup>. Participants were given either  $2 \times 10^{11}$  *B. longum* and 6 g of prebiotic fructo-oligosaccharide/inulin mix ( $n = 9$ ) or placebo ( $n = 9$ ) twice a day for 4 wk. The results showed that consumption of symbiotic treatment over 4 wk significantly ameliorated mucosal mRNA levels of human beta-defensins 2, 3, and 4, which are strongly upregulated in active UC. In addition, mucosal mRNA levels of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and IL-1 $\alpha$  were significantly decreased in the symbiotic group, with remission of sigmoidoscopy scores. This small trial provided the potential efficacy of symbiotic therapy for patients with active UC. The effect of *L. acidophilus* La-5 and *B. lactis* BB-12 (Probio-Tec AB-25) was investigated in a

randomized, double-blind, placebo-controlled trial<sup>[55]</sup>. Patients with left-side UC were given either Probio-Tec AB-25 ( $n = 20$ ) or placebo ( $n = 12$ ) for 52 wk, and clinical, endoscopic, and histological parameters were analyzed at entry and in case of relapse. In this study, there was no statistical difference in remission rate and the median time to relapse between the two groups after 1 year of treatment. The authors concluded that Probio-Tec AB-25 was well tolerated by study participants and that a failure to prove efficacy in remission rate may be due to the small sample size.

The use of probiotics as maintenance therapy in UC patients seems promising. Two randomized, double blind, double-dummy clinical trials to determine the efficacy of EcN were conducted. In the preliminary study, 120 patients with inactive UC were treated with either an oral preparation of EcN containing  $25 \times 10^9$  bacteria daily or mesalazine 1500 mg daily for 12 wk. The start and end DAI scores demonstrated no significant difference between the two groups. The relapse rate was 16.0% in EcN group and 11.3% among those taking mesalazine<sup>[56]</sup>. The same authors investigated the effectiveness of EcN in 327 patients with UC. Subjects received either probiotics containing  $5 \times 10^{10}$  bacteria or mesalazine 1500 mg daily ( $n = 162, 165$ ) for 12 mo. According to the per protocol analysis, 36.4% of patients in the EcN group experienced a relapse, compared with 33.9% of patients in the mesalazine group. The investigators concluded that EcN is efficient and safe in maintaining remission, and equivalent to the standard mesalazine<sup>[57]</sup>. Another study also confirmed that the administration of EcN was as effective as mesalazine in the maintenance of remission in patients with UC<sup>[58]</sup>. Patients with active UC were randomized to EcN treatment of  $5 \times 10^{10}$  probiotics/d ( $n = 57$ ) or mesalazine treatment (1200 mg/d,  $n = 59$ ) for 12 mo. In the intention-to-treat analysis, the health conditions were improved in 39 (68.4%) subjects in the EcN group and 44 (74.5%) subjects in the mesalazine group. The mean time of remission was 42 d (median 37 d) in the EcN group and 44 d (mean 42 d) in the mesalazine group. These results support the conclusion that EcN treatment has an equivalent effect to mesalazine in maintaining remission of UC.

The efficacy of VSL3 on induction and maintenance of remission in patients with active UC was studied in a long-term, randomized, double-blind, placebo-controlled trial<sup>[59]</sup>. In this study, subjects were children (mean age: 9.8 years; range: 1.7-16.1 years) and they were received either VSL3 containing  $4.5-18 \times 10^{11}$  bacteria/d ( $n = 14$ ) or placebo ( $n = 15$ ) for 1 year. All of the 29 patients were also treated with mesalazine during this trial period. Three of the 14 (21.4%) subjects in the VSL3 group and 11 of the 15 (73.3%) subjects in the placebo group relapsed within 1 year of follow-up. At 6 mo, 12 mo, or at time of relapse, endoscopic and histological scores were significantly lower in the VSL3 group compared with the patients in the placebo group. No adverse effect related to VSL3 was observed. Although the sample size of this

study was small, it provided evidence for the efficacy and safety of VSL3 in pediatric UC patients receiving conventional IBD therapy.

Another study evaluated the efficacy of VSL3 supplementation in patients with mild-to-moderate UC who were already being treated with 5-aminosalicylic acid (5-ASA) and/or immunosuppressants<sup>[60]</sup>. The patients were randomly treated with either VSL3 ( $n = 65$ ) including  $3600 \times 10^9$  CFU/d or placebo ( $n = 66$ ) for 8 wk. After 8 wk, decreases in DAI scores of 50% or more were significantly higher in the VSL3 group than in the placebo group. However, there was no significant difference in stool frequency, physician rating of disease, and endoscopic scores between the two groups. Although the mechanism(s) of VSL3 treatment in IBD therapy is unclear, these two randomized, double-blind, placebo-controlled clinical trials showed the potential synergistic activity of probiotics.

A large number of animal model studies have reported protective effects of probiotics, and these results are summarized elsewhere<sup>[61]</sup>. Most of the studies used DSS mouse<sup>[62-64]</sup>, trinitrobenzene sulfonic acid (TNBS) mouse<sup>[65-67]</sup> and IL-10<sup>-/-</sup> mouse<sup>[68-70]</sup>. The most frequently used probiotics were VSL3, *Lactobacillus*, *Bifidobacterium*, and *Saccharomyces*. The proposed mechanisms of action were as follows: (1) reduction of inflammatory cytokine and chemokine levels; (2) downregulation of TLR signaling followed by immune suppression; (3) increase in epithelium gap junction protein expressions; (4) prevention of pathogen growth and/or attachment; (5) increase in mucin production; (6) decrease in leukotriene B4 production; and (7) increase in short-chain fatty acid production. The reduced level of inflammatory cytokines and chemokines were thought to be mediated through signal transducer and activator of transcription-3 signaling<sup>[71,72]</sup> and/or NF- $\kappa$ B activation<sup>[73,74]</sup>.

Although the specific mechanisms of action of probiotics are unknown, their therapeutic effects may be related to modulation of antigen-presenting cells. Local T cell immunity is an important factor involved in the specific intestinal immune system and its activation is programmed by dendritic cells (DCs) that help maintain mucosal tolerance and contribute to the development of chronic intestinal inflammation as key initiators of innate and adaptive immune responses in the gut<sup>[75-77]</sup>. However, the immunological mechanisms of certain probiotics are still poorly understood while several studies have demonstrated that anti-inflammatory activity of probiotics is due to modulation of DC and regulatory T cell (Treg) phenotype function. It was reported that the therapeutic effect of VSL3 was due to regulation mucosal CD4+ T cell responses in a colitis-associated CRC model<sup>[78]</sup>. VSL3 administration in UC patients significantly decreased TLR-2 expression on colonic DC<sup>[79]</sup>. Also, VSL3 supplementation increased IL-10 production and decreased IL-12p40 production by colonic DC<sup>[76]</sup>. VSL3 administration induced a significant expansion of mucosal Treg cells in UC patients<sup>[80]</sup>. *L. rhamnosus* induced maturation of monocyte-derived DC and induced both lower

IL-12 and IL-18 production and development of T cells without a typical Th phenotype<sup>[81]</sup>. A probiotic mixture containing *L. helveticus* and *L. rhamnosus* increased follicular Treg cells in *Citrobacter rodentium*-induced colitis<sup>[82]</sup>. *L. casei* inhibited TNF-induced secretion of the T-cell chemokine interferon-inducible protein 10 (IP-10) in IECs by blocking IP-10 protein secretion and IP-10-mediated T-cell transmigration<sup>[83]</sup>. *L. casei* lysate DN-114 001 treatment increased the numbers of CD4(+)Foxp3(+)Treg cells in mesenteric lymph nodes, decreased the production of TNF- $\alpha$ , interferon- $\gamma$ , and IL-10 in Peyer's patches and large intestine, and changed the gut microbiota composition in a DSS-induced colitis model<sup>[84]</sup>. *L*-peptidoglycan purified from Ls33 also ameliorated TNBS-induced colitis by development of CD103(+) DCs and CD4(+)Foxp3(+) Treg cells<sup>[85]</sup>. Thomas *et al*<sup>[86]</sup> showed that *Saccharomyces boulardii*, a probiotic yeast preparation, reduced IL-6 and TNF- $\alpha$  and decreased the expression of co-stimulated surface markers CD40, CD80, and the migration marker CD197 (CCR7) on LPS-stimulated human 92 induced apoptosis of antigen-stimulated T cells. These data suggest that each strain exhibits a different ability to modulate DC and Treg functions and explain various potential mechanism(s) of bacterial strains.

Current evidence suggests that probiotics are good complementary and alternative medicine candidates to maintain remission and prevent relapse of UC. However, the clinical efficacy results are limited, and strain-specific mechanistic explanations are insufficient. Additional large-scale clinical trials are required to shed light on optimal doses and treatment periods.

### Antioxidant vitamins and phytochemicals

Oxidative stress is known as a potential etiological and/or triggering factor in the initiation and preservation of UC<sup>[47,87-89]</sup>. Inflammation augments oxidative stress by activating reactive oxygen and/or reactive nitrogen, generating enzymes such as NAD(P)H oxidase, inducible nitric oxide synthase, and myeloperoxidase<sup>[89,90]</sup>. A group of antioxidants has been used to ameliorate the clinical condition of UC patients. In addition, several studies have shown that patients with UC often have antioxidant nutrient deficiencies at the time of diagnosis<sup>[91-93]</sup>. Although the important role of antioxidants in UC has been seen in several studies, few clinical trials have been conducted. A randomized, controlled trial evaluated the efficacy of a nutritionally balanced oral supplement including fish oil, fructo-oligosaccharides, gum arabic, vitamin E, vitamin C, and selenium in 121 patients with mild-to-moderate UC<sup>[93]</sup>. Patients consumed either 510.3 g of the oral supplement or placebo each day for 6 mo. Compared with the placebo group, both intent-to-treat and completer patients given the oral supplement showed a significant decrease in the dose of prednisone required to control clinical symptoms over 6 mo. Mirbagheri *et al*<sup>[94]</sup> performed an open-label, preliminary trial for the efficacy of D- $\alpha$ -tocopherol using 14 patients who were receiving concomitant therapy with 5-ASA and/or immunosuppressants. Patients received a D- $\alpha$ -

**Table 1** Action targets of major beneficial dietary components in ulcerative colitis

	<b>Omega-3 fatty acids</b>	<b>Probiotics</b>	<b>Anti-oxidants</b>	<b>Aloe</b>
Beneficial bacteria growth		+		
Short chain fatty acid production		+		
Mucin repletion		+		+
Junction protein restoration		+		
Suppression of inflammatory eicosanoids	+			
Blockade of inflammatory signals	+		+	+
Suppression of immune response	+	+	+	
Reduction in oxidative stress			+	

tocopherol enema (8000 U/d) for 12 wk. At the end of 12 wk, the DAI score had statistically decreased from the beginning of the study, all 14 patients responded clinically to the therapy, and remission was induced in 9 patients (64%) without adverse events.

In recent years, several studies have investigated the efficacy of curcumin, a polyphenolic antioxidant in *Curcuma longa* L., in experimental models of UC. These studies have showed a strong antioxidant effect, as well as an anti-inflammatory effect, of curcumin in this model. A small, open-label, pilot clinical trial of the effect of curcumin in patients with IBD (5 UC, 5 CD) receiving IBD medication (5-ASA or corticosteroids) was conducted<sup>[95]</sup>. The patients were given curcumin 1100 mg/d for 1 mo and 1650 mg/d for an additional 2 mo. All 5 patients with UC had significant improvement in their medication, as follows: two patients stopped taking 5-ASA, two reduced 5-ASA dosage, and one stopped corticosteroids entirely. This encouraging pilot study showed the strong potential efficacy of curcumin in UC patients.

In a multicenter, randomized, double-blind, placebo-controlled trial, the efficacy of curcumin in 89 patients with quiescent UC was evaluated<sup>[96]</sup>. In addition to their usual medication (sulfasalazine or mesalamine), participants received either curcumin (2 g/d) or placebo ( $n = 45, 44$ ) for 6 mo. DAI and endoscopic scores were determined at entry, every 2 mo (DAI), at the conclusion of the 6-mo trial, and after a 6-mo follow-up. The relapse rate was significantly lower in the curcumin group than in the placebo group and the recurrence rate was significantly reduced in the curcumin group compared with the placebo group. Both the DAI and endoscopic score were also improved in the curcumin group without any adverse effects. Although only two clinical trials have been performed, the potential therapeutic capability of curcumin in UC patients has been shown, possibly resulting from its antioxidant and anti-inflammatory effects. However, large-scale, randomized, double-blind, placebo-controlled clinical trials are required to achieve convincing evidence for the routine use of antioxidants in patients with UC.

Many studies have reported that antioxidant supplementation has therapeutic efficacy in animal models, and these results were summarized in a recent review<sup>[97]</sup>.

Mechanisms of action include: (1) direct scavenging of reactive oxygen species; (2) suppression of proinflammatory protein expression by downregulating related enzymes or transcription factors; and (3) alteration of leukocyte cell surface molecules.

UC is associated with increased gastrointestinal permeability<sup>[98,99]</sup>. Although the causes of increased permeability are not completely understood, an inflammation-associated increase in permeability has been shown to result in bacterial translocation into the lamina propria, which exacerbates the loss of barrier function. We have previously reported that aloe anthraquinones and chromone ameliorate colonic inflammatory responses in a DSS-induced UC model; aloein, an aloe chromone, showed the most drastic results<sup>[100]</sup>. Additionally, we studied whether aloein modulates mucosal permeability and found that this phytochemical recovered the gene expression levels of apical junctional complex proteins, which regulate gut permeability and barrier function (unpublished data). These results suggest that the anti-inflammatory property of aloein is partly based on regulation of gut permeability, although human studies of clinical use are required.

Over the last few decades, phytochemical-based complementary and alternative treatments have emerged as relatively effective and safe therapeutic strategies for UC. Although a significant number of animal studies have established the potential beneficial effects on UC of phytochemicals and bioactive components of other natural sources, the exact mechanisms and/or molecular targets of their anti-inflammatory actions are not fully understood. Further studies are needed to evaluate their safety, targets, phytochemical-drug interactions, and pharmacokinetic information. Understanding these issues through well-designed clinical trials will enable us to choose reliable complementary and alternative therapies for patients with UC.

## CONCLUSION

Because the increased prevalence of UC is potentially due to changes in dietary habits, especially in Eastern countries, it is now considered a lifestyle-related disease. It has been proposed that UC onset and progress are modulated by dietary factors such as excess energy intake, saturated fat intake, fatty acid ratio in the diet, and antioxidant intake. However, well-controlled human intervention trials are required to clarify the effect of nutritional factors on UC development. As understanding of the mechanisms involved in UC improves, a target-oriented search for compounds possessing therapeutic or complementary value becomes possible (Table 1). Nutritional modulation that aims to not only prevent the onset and relapse of the disease but also maintain remission will contribute to the successful management of UC.

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## Vitamin B6 and colorectal cancer: Current evidence and future directions

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colorectal cancer comparing high with low concentrations. The reasons for the discrepancy in the results between dietary-based and plasma-based studies remain unresolved. Other unresolved questions include the effects of vitamin B6 intake in early life (*i.e.*, childhood or adolescence) and of suboptimal vitamin B6 status on colorectal cancer risk, whether the associations with vitamin B6 differ across molecular subtypes of colorectal cancer, and whether the vitamin B6-colorectal cancer association is modified by genetic variants of one-carbon metabolism.

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### Abstract

Colorectal cancer remains the third most common cancer in both women and men worldwide. Identifying modifiable dietary factors is crucial in developing primary prevention strategies. Vitamin B6 is involved in more than 100 coenzyme reactions, and may influence colorectal cancer risk in multiple ways including through its role in one-carbon metabolism related DNA synthesis and methylation and by reducing inflammation, cell proliferation, and oxidative stress. Observational studies of dietary or dietary plus supplementary intake of vitamin B6 and colorectal cancer risk have been inconsistent with most studies reporting non-significant positive or inverse associations. However, published studies of plasma pyridoxal 5'-phosphate (the active form of vitamin B6) levels consistently support an approximately 30%-50% reduction in risk of

### INTRODUCTION

Colorectal cancer is the third most common cancer worldwide<sup>[1]</sup>. In the United States, colorectal cancer will account for approximately 143 460 new cases in 2012<sup>[2]</sup>. The wide variation in age-adjusted incidence rates for colorectal cancer between countries suggests a role of environmental factors such as diet in colorectal carcinogenesis. However, as summarized in several recent reviews<sup>[3-5]</sup>, the effect of dietary factors on colorectal cancer remains largely inconclusive. For example, based on a comprehensive review of published studies, the 2011 World Cancer Research Foundation and American Institute for Cancer Research report on diet and colorectal



cancer identified that there was convincing evidence only for red meat, processed meat, and alcoholic drinks (in men) as risk factors and for dietary fiber as a protective factor for colorectal cancer<sup>[3]</sup>. The effect of other dietary factors such as vitamin B6 on colorectal cancer remains to be elucidated. Vitamin B6, a one-carbon metabolism related nutrient, may have a potential role in colorectal carcinogenesis. The current epidemiologic studies examining vitamin B6 and risk of sporadic colorectal cancer and adenomas are briefly summarized here. Although the possible effect of vitamin B6 may differ by molecular subtype of colorectal cancer or be mediated by genetic variants in one-carbon metabolism, few data are available to date<sup>[6-11]</sup> and thus are not reviewed here.

Vitamin B6 is a water-soluble vitamin that participates in more than 100 coenzyme reactions involved in the metabolism of protein, carbohydrates, and lipids<sup>[12]</sup>. In the United States, the major food sources for vitamin B6 include fortified cereals, starchy vegetables, beef, and poultry<sup>[12]</sup>. The recommended daily allowance (RDA) for vitamin B6 intake is 1.7 mg/d for men and 1.5 mg/d for women aged 51 years or older<sup>[12]</sup> although some subgroups of the population including smokers, blacks, seniors, and current and former oral contraceptive users require higher intakes<sup>[13]</sup>. Plasma pyridoxal 5'-phosphate (PLP) is the active form of vitamin B6 and is most commonly used to measure vitamin B6 status. A PLP level of more than 20 nmol/L is an indicator of adequate vitamin B6 status in adults<sup>[12]</sup>. The 2003-2006 national health and nutrition examination survey found that 11% of vitamin B6 supplement users (approximately 20%-30% of United States adults<sup>[14]</sup>) and 24% of people in the United States who do not take supplements containing vitamin B6 have suboptimal plasma PLP concentrations (< 20 nmol/L)<sup>[13]</sup>.

## VITAMIN B6 AND POTENTIAL MECHANISMS RELATED TO COLORECTAL CARCINOGENESIS

Vitamin B6 may influence colorectal carcinogenesis through its role in DNA synthesis and methylation<sup>[15]</sup>, both of which are potentially involved in colorectal carcinogenesis. In addition, animal models have demonstrated that supplemental vitamin B6 suppressed cell proliferation and reduced the number of tumors in the colon<sup>[16,17]</sup>. Moreover, vitamin B6 has been shown to inhibit angiogenesis<sup>[18]</sup>, suppress nitric oxide<sup>[16]</sup>, and reduce oxidative stress<sup>[19]</sup>, all of which are associated with preventing carcinogenesis. Further, low vitamin B6 status may link to chronic inflammation<sup>[20]</sup>, a potential risk factor for colorectal cancer<sup>[21]</sup>, based on significantly lower PLP concentrations observed among patients with inflammatory bowel diseases<sup>[22]</sup> and rheumatoid arthritis<sup>[23,24]</sup> compared to generally healthy populations. Other evidence of the link between vitamin B6 and inflammation includes the inverse relation of plasma PLP with cardiovascular disease<sup>[25,26]</sup>, C-reactive protein<sup>[23,27]</sup>, and tumor necrosis factor alpha<sup>[24]</sup>.

## OBSERVATIONAL STUDIES OF VITAMIN B6 INTAKE AND COLORECTAL CANCER RISK

Despite potential mechanisms supporting the hypothesis that vitamin B6 may reduce colorectal cancer risk, epidemiologic evidence examining vitamin B6 intake and colorectal cancer risk has been inconclusive. At least 9 case-control studies have examined the relation between vitamin B6 intake and colorectal cancer risk. The majority of the case-control studies reported a modest significant inverse association for comparisons of the highest with the lowest vitamin B6 intake categories. For example, a quantitative review of six case-control studies published in 2008 reported a summary multivariable relative risk (RR) of 0.67 (95%CI: 0.60-0.75) comparing high with low vitamin B6 intake<sup>[28]</sup>. However, there was borderline significant heterogeneity among these case-control studies (*P* value for heterogeneity = 0.09) with risk estimates ranging from 0.51 to 1.00. In addition, a meta-analysis of nine cohort studies that included eleven risk estimates (3 studies analyzed men and women separately) found no substantial effect of vitamin B6 intake on colorectal cancer risk (high *vs* low intake categories, summary RR = 0.90, 95%CI: 0.75-1.07). However, there was statistically significant heterogeneity in the results from the cohort studies (*P* value for heterogeneity = 0.01) with 6 cohort studies reporting 18%-39% lower risks of colorectal cancer comparing the highest *vs* lowest categories (the associations in three studies were statistically significant) and 5 studies reporting nonsignificant positive associations<sup>[29]</sup>. In the meta-analysis, only two cohort studies had evaluated associations with total vitamin B6 intake and a non-significant association was observed (summary RR = 0.90, 95%CI: 0.73-1.11)<sup>[29]</sup>. The only subsequently published study of two cohorts examined potential latency effects of vitamin B6 intake on colorectal cancer risk and found no difference for intakes measured 0-4 years before diagnosis compared to intakes measured 12-16 years before diagnosis<sup>[30]</sup>. Of note, the study populations in these studies were relatively well nourished, with a low prevalence (*i.e.*, 5%-10%) of individuals below the RDA levels of vitamin B6 intake, limiting the ability to test the potential effect of suboptimal vitamin B6 status on colorectal cancer risk.

## NESTED CASE-CONTROL STUDIES OF PLASMA PLP CONCENTRATIONS AND COLORECTAL CANCER RISK

Four out of five nested case-control studies conducted to date have shown that higher pre-diagnostic plasma PLP concentrations were statistically significantly associated with a 30%-50% lower risk of colorectal cancer<sup>[29]</sup>. The first study was from the United States (*n* = 188 cases, highest *vs* lowest quartile, RR = 0.48, 95%CI: 0.25-0.92, *P* value for trend = 0.03)<sup>[31]</sup>. The second study conducted

in Finland found that men in the highest quartile of PLP concentrations had non-significant lower risk of colorectal cancer ( $n = 275$  cases,  $RR = 0.61$ , 95%CI: 0.32-1.14,  $P$  value for trend = 0.08)<sup>[32]</sup>. Similar magnitudes of inverse associations were also observed in subsequent analyses using data from the Multiethnic Cohort study ( $n = 223$  cases,  $RR = 0.52$ , 95%CI: 0.29-0.92,  $P$  value for trend = 0.03)<sup>[33]</sup> and the Physicians' Health Study ( $n = 197$  cases,  $RR = 0.49$ , 95%CI: 0.26-0.92,  $P$  value for trend = 0.01)<sup>[34]</sup>. Likewise, in the largest and most recent analysis to date ( $n = 1365$  cases), the  $RR$  comparing the highest to lowest quintile was 0.68 (95%CI: 0.53-0.87,  $P$  value for trend < 0.001) in the European Prospective Investigation into Cancer and Nutrition cohort<sup>[9]</sup>. As shown in the meta-analysis of these studies<sup>[29]</sup>, the pooled  $RR$  of colorectal cancer for the highest vs lowest categories of PLP levels was 0.52 (95%CI: 0.38-0.71).

## CLINICAL TRIALS OF VITAMIN B6 SUPPLEMENT AND COLORECTAL CANCER

The effect of treatment with vitamin B6 supplements (40 mg/d) on colorectal cancer incidence and mortality has been evaluated, to the best of our knowledge, in only two randomized double-blind, placebo-controlled trials, the Norwegian Vitamin Trial<sup>[35]</sup> and the Western Norway B Vitamin Intervention Trial<sup>[36]</sup>. These studies were not primarily designed to examine cancer outcomes and included 6837 participants with ischemic heart disease after a median of 39 mo of treatment and an additional 38 mo of post-trial observational follow-up<sup>[37]</sup>. In both trials, participants were randomized into one of four groups: (1) folic acid (0.8 mg/d), vitamin B12 (0.4 mg/d), and vitamin B6 (40 mg/d); (2) folic acid (0.8 mg/d) and vitamin B12 (0.4 mg/d); (3) vitamin B6 alone (40 mg/d); or (4) placebo. The pooled analysis of data from these two trials showed no benefit of vitamin B6 supplementation on incident colorectal cancer or fatal colorectal cancer. Of note, only a limited number of colorectal cancer cases and deaths were included in the analysis. A total of 26 participants (1.5%) who received vitamin B6 and 22 (1.3%) participants in the placebo group were diagnosed with incident colorectal cancer during the trial (vitamin B6 vs non-vitamin B6 group,  $RR = 1.18$ , 95%CI: 0.69-2.00)<sup>[37]</sup>. Furthermore, there were only 5 deaths due to colorectal cancer in the vitamin B6 group and 7 in the placebo group (vitamin B6 vs non-vitamin B6 group,  $RR = 0.51$ , 95%CI: 0.17-1.55)<sup>[37]</sup>.

## VITAMIN B6 INTAKE, PLASMA PLP CONCENTRATIONS AND COLORECTAL ADENOMAS

The evidence for the association between vitamin B6 intake or plasma PLP concentrations and risk of colorectal adenoma, precursors of colorectal cancer, is less con-

sistent than observed for colorectal cancer, with only a small number of studies published<sup>[10,11,31,38]</sup>. Specifically, the first study found a suggestive inverse association between plasma PLP concentration and advanced ( $\geq 1$  cm in size, or villous or tubulovillous) distal colorectal adenoma ( $n = 408$  cases,  $RR = 0.65$ , 95%CI: 0.37-1.11,  $P$  value for trend = 0.08), but a weaker association with low risk of (small and tubulovillous) adenoma ( $n = 210$  cases,  $RR = 0.85$ , 95%CI: 0.52-1.38,  $P$  value for trend = 0.52)<sup>[31]</sup>. The other cohort study showed that high plasma levels of PLP were inversely associated with risk of colorectal adenoma ( $n = 210$  cases, highest vs lowest tertile,  $RR = 0.44$ , 95%CI: 0.26-0.74,  $P$  value for trend = 0.002)<sup>[10]</sup>. Among 2 studies that evaluated the effect of vitamin B6 and adenoma recurrence, the first study from the Wheat Bran Fiber intervention trial showed a lower odds of adenoma recurrence for higher vitamin B6 intake ( $n = 495$  recurrences, highest vs lowest quartile,  $OR = 0.65$ , 95%CI: 0.45-0.94,  $P$  value for trend = 0.03)<sup>[38]</sup>. The Aspirin/Folate Polyp Prevention Study, a trial of folic acid supplementation, found a borderline significant inverse association with plasma PLP concentrations and risk of adenoma recurrence ( $n = 430$  recurrences, highest vs lowest quartile,  $RR = 0.78$ , 95%CI: 0.61-1.00,  $P$  value for trend = 0.08)<sup>[11]</sup>.

## DISCUSSION

Overall, based on a meta-analysis of nine cohort studies a substantial effect of vitamin B6 intake in adulthood and colorectal cancer risk was not evident although the study-specific results were inconsistent. In contrast, all five studies of circulating PLP levels found that participants with higher plasma PLP levels had a 30%-50% lower risk of colorectal cancer with the associations being statistically significant in four of the studies. Of note, only two nested case-control studies<sup>[31,33]</sup> have examined associations with both vitamin B6 intake and PLP levels and colorectal cancer risk and in these two studies inverse associations of similar magnitude were observed for vitamin B6 intake and plasma PLP concentrations and colorectal cancer risk. Several issues related to examining associations between vitamin B6 and colorectal cancer risk are discussed below.

### Confounding by other factors?

Vitamin B6 intake is an important determinant of PLP levels<sup>[39]</sup>. However, individuals with high vitamin B6 intake tend to have healthy behaviors such as higher physical activity, less smoking, and higher intakes of folate, calcium, and vitamin D compared to individuals with lower vitamin B6 intake<sup>[30,40-42]</sup>. Because being physically active, not smoking, and having higher intakes of folate, calcium and vitamin D may reduce the risk of colorectal cancer<sup>[3,5,43,44]</sup>, the inverse associations observed with higher PLP concentrations might be simply due to the correlations between vitamin B6 intake and these healthy behaviors. Although previous studies have adjusted for these potential confounding factors<sup>[29,30]</sup>, residual confounding may still exist. Further, it is challenging to tease

out the independent effect of vitamin B6 from certain other nutrients such as folate, calcium, and vitamin D given that their intakes are positively correlated, particularly for intakes from food and supplemental sources combined. In addition, variation in plasma PLP could possibly reflect metabolic states such as chronic inflammation<sup>[22-24,27,45]</sup>, a risk factor for colorectal cancer<sup>[21]</sup>. Studies have shown that patients with inflammatory bowel diseases<sup>[22]</sup> and rheumatoid arthritis<sup>[23,24]</sup> have significantly lower plasma PLP concentrations than healthy individuals. Although the data are not in full agreement<sup>[46]</sup>, plasma PLP concentrations also have been found to be inversely correlated with C-reactive protein levels<sup>[23,27]</sup>, and tumor necrosis factor alpha levels<sup>[24]</sup>, both of which are markers of inflammation and possible risk factors for colorectal cancer<sup>[47]</sup>. Possibly, lower PLP levels could reflect a patho-physiologic state such as inflammation, which may be associated with higher risk of colorectal cancer, but increasing plasma PLP concentrations through increased intake may not necessarily lead to lower colorectal cancer risk. However, one study found that the inverse association with higher plasma PLP concentrations did not change even after adjustment for plasma concentrations of homocysteine, C-reactive protein, tumor necrosis factor alpha, and interleukin-6<sup>[34]</sup>. Nonetheless, it is unclear whether plasma PLP levels *per se* or the healthy behaviors or physiologic states associated with plasma PLP levels conferred the benefits observed in the studies of PLP concentrations and colorectal cancer risk.

#### Measurement error in assessment of vitamin B6?

Measurement error may have occurred in estimated vitamin B6 intake assessed using food frequency questionnaires (FFQs). However, the relatively high correlations (ranged from 0.4 to 0.8) observed between vitamin B6 intake assessed by FFQs and intake assessed using a reference methods (*i.e.*, dietary records)<sup>[6,30,40-42,48,49]</sup> reduce the possibility of missing a strong association between vitamin B6 intake and colorectal cancer risk in cohort studies. Moreover, total vitamin B6 intake (from food and supplemental sources combined) appears to be a good predictor of plasma PLP concentrations. For example, among representative random samples from the Nurses' Health Study ( $n = 381$  women) and the Health Professionals Follow-up Study ( $n = 345$  men) who had provided blood samples and served as controls in a nested case-control study of colorectal cancer<sup>[30]</sup>, an approximately 3-fold difference was observed in mean plasma PLP concentrations comparing the top vs bottom quintiles of total vitamin B6 intake. Specifically, for the top and bottom quintile categories of total vitamin B6 intake, the mean plasma PLP concentrations were 98.3 nmol/L and 38.9 nmol/L in women and 183.2 nmol/L and 66.0 nmol/L in men. In addition, the Spearman correlation coefficients between total vitamin B6 intake and plasma PLP concentrations were 0.52 in women and 0.54 in men<sup>[30]</sup>. Thus, the ability of FFQs to predict an almost 3-fold difference in plasma PLP levels argues against measurement error in vitamin B6 intake masking detection of a strong associa-

tion between vitamin B6 intake and colorectal cancer risk. Misclassification of vitamin B6 status also may occur in studies of PLP levels and colorectal cancer risk given that the studies published to date have only used one blood sample to measure PLP levels which may not reflect long term vitamin B6 status.

#### Interaction with other factors?

Inconsistent results might also result from differences in distributions of potential effect modifiers of the association between vitamin B6 and colorectal cancer risk. Given that alcohol consumption may decrease vitamin B6 levels<sup>[50,51]</sup>, any effect of vitamin B6 on colorectal cancer risk might therefore be stronger among heavy drinkers. To date, results have been conflicting among the six studies we identified. A non-significant interaction between vitamin B6 and total alcohol consumption including wine, beer, spirits was observed in three studies of vitamin B6 intakes<sup>[30,52]</sup> and one study of plasma PLP levels<sup>[31]</sup>. In contrast, a stronger inverse association with plasma PLP concentrations<sup>[9]</sup> or vitamin B6 intake<sup>[41]</sup> has been reported among alcohol drinkers compared to nondrinkers. In addition, if vitamin B6 influences the development of colorectal cancer through the one-carbon metabolism pathway, the potential benefit of vitamin B6 might be stronger among individuals with low intake of other one-carbon metabolism related nutrients such as folate, riboflavin, methionine, and vitamin B12. However, current studies are limited and found no evident pattern<sup>[30,31]</sup>. With respect to adenomas, studies are limited and results have also been mixed. Results from the Multi-ethnic Cohort Study showed a non-significant interaction between plasma PLP and alcohol consumption<sup>[10]</sup> and the Aspirin/Folate Polyp Prevention Study found that the inverse association with plasma PLP was evident only among nondrinkers ( $P$  value for interaction = 0.03)<sup>[11]</sup>.

#### Timing of intake is important?

Because most cohort studies conducted to date have only a single assessment of vitamin B6 intake<sup>[29,30]</sup>, it is uncertain when in the natural history vitamin B6 intake may influence colorectal cancer risk. However, a recent study that specifically evaluated the timing using time lagged analyses found no clear pattern<sup>[30]</sup>. In contrast, when potential latency effects of total folate intake (another one-carbon metabolism related nutrient) were examined, only total folate intake measured 12-16 years prior to diagnosis of colorectal cancer was significantly associated with a lower risk of colorectal cancer, consistent with the only prior study examining the timing of folate intake on colorectal cancer risk<sup>[53]</sup>. When total folate and total vitamin B6 intakes measured 12-16 years prior to diagnosis were simultaneously included in the multivariate model, a statistically significant inverse association continued to be observed for total folate intake while a weak, non-significant association was observed for total vitamin B6 intake. However, it is unknown whether vitamin B6 intake in childhood, adolescence, or early adulthood might be important in colorectal carcinogenesis because dietary



habits experienced early in development may play an important role in adult disease<sup>[54,55]</sup>. The previous observational studies that we identified have focused on vitamin B6 intake in adulthood<sup>[30]</sup>.

## CONCLUSION

In contrast to the inverse associations observed in most studies of plasma PLP concentrations, the results from cohort studies of vitamin B6 intake in adulthood and colorectal cancer risk in relatively nourished populations have been inconsistent. The reason for this discrepancy between dietary-based and plasma-based studies remains unresolved and a better understanding is needed of the determinants of plasma PLP concentrations. Future studies should focus on early life intake (*i.e.*, childhood or adolescence), the effects of suboptimal vitamin B6 status, molecular subtypes of colorectal cancer, and effect modification by genetic variants of one-carbon metabolism.

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## Dietary flavonoid intake and risk of stomach and colorectal cancer

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### Abstract

Stomach and colorectal cancers are common cancers and leading causes of cancer deaths. Because the alimentary tract can interact directly with dietary components, stomach and colorectal cancer may be closely related to dietary intake. We systematically searched published literature written in English *via* PubMed by searching for terms related to stomach and colorectal cancer risk and dietary flavonoids up to June 30, 2012. Twenty-three studies out of 209 identified articles were finally selected for the analysis. Log point effect estimates and the corresponding standard errors were calculated using covariate-adjusted point effect estimates and 95% CIs from the selected studies. Total dietary flavonoid intake was not associated with a reduced risk of colorectal or stomach cancer [odds ratio (OR) (95%CI) = 1.00 (0.90-1.11) and 1.07 (0.70-1.61), respectively]. Among flavonoid subclasses, the intake of flavonols, flavan-3-ols, anthocyanidins, and proanthocyanidins showed a significant inverse association with colorectal cancer risk [OR (95%CI) = 0.71 (0.63-0.81), 0.88

(0.79-0.97), 0.68 (0.56-0.82), and 0.72 (0.61-0.85), respectively]. A significant association was found only between flavonols and stomach cancer risk based on a limited number of selected studies [OR (95%CI) = 0.68 (0.46-0.99)]. In the summary estimates from case-control studies, all flavonoid subclasses except flavones and flavanones were inversely associated with colorectal cancer risk, whereas neither total flavonoids nor any subclasses of flavonoids were associated with colorectal cancer risk in the summary estimates based on the cohort studies. The significant association between flavonoid subclasses and cancer risk might be closely related to bias derived from the case-control design. There was no clear evidence that dietary flavonoids are associated with reduced risk of stomach and colorectal cancer.

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**Key words:** Flavonoids; Flavonols; Flavones; Flavanones; Flavan-3-ols; Anthocyanidins; Proanthocyanidins; Cancer risk; Meta-analysis

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### INTRODUCTION

Stomach and colorectal cancer are common cancers and leading causes of cancer deaths<sup>[1]</sup>. Because the alimentary tract can interact directly with dietary components, stomach and colorectal cancer may be closely related to dietary intake. The consumption of meat, especially of red and processed meats that produce N-nitrosamine, was positively associated with stomach and colorectal cancer risk in a previous meta-analyses<sup>[2-4]</sup>. However, fruit and vegetable intakes were inversely associated with

colorectal cancer risk in people with high intakes of red and processed meat<sup>[5]</sup>, and inverse association between vitamin C intake and stomach cancer risk was stronger in high red and processed meat intake group<sup>[6]</sup>. High fruit and vegetable intakes are associated with beneficial health effects, and these effects have been partly attributed to the high flavonoid content of these foods. Flavonoids are polyphenolic compounds that are abundant in fruits and vegetables. The beneficial health effects of flavonoids have been attributed to their free radical scavenging properties. In addition to their antioxidant properties, flavonoids have antiviral, antiallergic, antiinflammatory, and antitumor activities<sup>[7,8]</sup>. Flavonoids are generally classified as flavonols, flavones, flavanones, flavanols, isoflavones or anthocyanidins based on their chemical structure<sup>[9-11]</sup>. The antioxidant activities of the dietary flavonoids and their subclasses vary due to the differences in their chemical structures. Flavonols, the most common flavonoids in foods, include kaempferol, myricetin, quercetin, and isorhamnetin. Among them, quercetin is abundant in onions and apples, and has been studied extensively due to its high bioavailability and strong antioxidant effects. Flavones, such as apigenin and luteolin, are abundant in green leafy spices (*e.g.*, parsley, thyme, and celery), and the flavanones naringenin, hesperetin, and eriodictyol are found in citrus fruits. Flavanols, often referred to as flavan-3-ols, include catechin, gallic acid, and epicatechin, which are abundant in teas, red wine, and apples. Isoflavones, especially genistein, are found in soy foods and have been studied widely due to their antitumor properties. Anthocyanidins are found in cherries, strawberries, and red wine.

A reduced risk of cardiovascular disease associated with flavonoid intake has been observed in many epidemiological studies<sup>[12,13]</sup>. Flavonoids have been suggested to reduce the risk of cardiovascular disease by modulating various mechanisms<sup>[14]</sup>. However, the association between cancer risk and dietary flavonoid intake has less supportive evidence from epidemiological studies, and the results have been inconsistent. To date, meta-analyses have focused mainly on dietary flavonoids and cardiovascular disease<sup>[12,13]</sup> or tea flavonoids and lung cancer<sup>[15,16]</sup>. Thus, we performed a meta-analysis of summary data to calculate the effect estimates of dietary flavonoid intake, including individual subclasses of flavonoids, on stomach and colorectal cancer risk.

## DIETARY FLAVONOID INTAKE AND RISK OF STOMACH AND COLORECTAL CANCER

English-language studies were systemically searched using PubMed with the phrase “cancer risk and (stomach or gastric or colorectal or colon or bowel or rectal) and (flavonoid or flavonol or quercetin or kaempferol or myricetin or isorhamnetin or flavone or luteolin or apigenin or flavanone or eriodictyol or hesperetin or

naringenin or flavan-3-ol or catechin or epicatechin or theaflavin or anthocyanidin or cyanidin or delphinidin or malvidin or pelargonidin or peonidin or petunidin or proanthocyanidin or isoflavone)” up to June 30, 2012. The inclusion criteria were as follows: (1) original articles with a case-control or cohort design; (2) articles reporting intake of either total flavonoids or subclasses of flavonoids and stomach or colorectal cancer; (3) articles with at least 3 categories of dietary flavonoid intake; and (4) studies reporting adjusted odds ratios (OR) or relative risks (RR) with 95% CIs for the risk of stomach or colorectal cancer in subjects with the highest dietary flavonoid intake compared with those with the lowest intake of dietary flavonoids.

The retrieved studies were reviewed independently by two investigators (Woo HD and Kim J). Data on authors, publication year, types of flavonoids, cancer sites, country in which the study was performed, number of cases and controls, categories of flavonoids or subclasses of flavonoids, subclasses included in the calculation of total flavonoids, and adjusted OR/RR and 95% CI were collected for the meta-analysis. The multivariate-adjusted values for OR/RR and 95% CI were selected for the meta-analysis to reduce the effects of potential confounding variables. If there was additional adjustment for fruit and vegetable intake, the adjusted values were selected.

All statistical analyses were performed using the STATA software package (version 10; Stata Corp, College Station, TX, United States). Log point effect estimates and the corresponding standard errors were calculated using the covariate-adjusted point effect estimates and 95% CI from selected studies and weighted by the inverse variance to calculate the summary estimates<sup>[17]</sup>. The heterogeneity across studies was measured using the *Q*-test based on the  $\chi^2$  statistic. Heterogeneity was considered statistically significant when  $P < 0.1$  for the *Q*-test and was quantified using the  $I^2$  test as described by Higgins *et al.*<sup>[18]</sup>. Based on the heterogeneity of the included studies, fixed or random effects models were selected to calculate the pooled effect measures. Sensitivity analyses were performed to test the robustness of the results of the combined effects.

A total of 209 studies were identified, and 160 studies were excluded based on the titles and abstracts. The full texts of the remaining 49 studies were reviewed, and 25 studies were excluded for the following reasons: 7 studies were not related to dietary flavonoid intake or any of the flavonoid subclasses; 5 studies presented only recurrence or survival analysis data; 5 studies were not related to stomach or colorectal cancer: 4 studies did not report cancer risk; 2 studies were review articles; and 2 studies reported plasma concentrations instead of dietary flavonoid intake.

Sensitivity tests were conducted for the remaining 24 studies of total flavonoids and each subclass of flavonoids. One study showing substantial influence on the summary estimates in the sensitivity tests was excluded<sup>[19]</sup>. Thus, 23 studies, comprising 13 case-control

studies and 10 cohort studies, were finally selected to estimate the overall effects of total dietary flavonoid or flavonoid subclass intake on stomach and colorectal cancer risk (Table 1)<sup>[20-42]</sup>. Begg's funnel plot and Egger's test were used to evaluate the publication bias in studies of total flavonoids and each subclass of flavonoids. Egger's test showed no significant bias.

The summary estimates for the risk of cancer in subjects with the highest dietary flavonoid intake, compared with that in subjects with the lowest flavonoid intake, are presented in Table 2. The heterogeneity was low in the overall results, but heterogeneity was found in several summary estimates of the studies that included mostly case-control design. Total dietary flavonoids were not associated with a reduced risk of colorectal or stomach cancer [OR (95%CI) = 1.00 (0.90-1.11) and 1.07 (0.70-1.61), respectively]. However, flavonol, flavan-3-ol, anthocyanidin, and proanthocyanidin intakes showed a significant inverse association with colorectal cancer risk among flavonoid subclasses [OR (95%CI) = 0.71 (0.63-0.81), 0.88 (0.79-0.97), 0.68 (0.56-0.82), and 0.72 (0.61-0.85), respectively]. A significant association was found only for flavonol intake and stomach cancer risk in a limited number of selected studies [OR (95%CI) = 0.68 (0.46-0.99)].

Subgroup analyses by study design or sex were conducted for each subclass of flavonoids. In case-control studies, all flavonoid subclasses except flavones and flavanones were inversely associated with colorectal cancer risk. However, neither total flavonoids nor any subclasses of flavonoids were associated with colorectal cancer risk in the cohort studies. Colorectal cancer showed a statistically significant association for the summary estimate of flavonols in both female and male subjects [OR (95%CI) = 0.84 (0.75-0.93) and 0.87 (0.79-0.96), respectively] and isoflavones in male subjects [OR (95%CI) = 0.90 (0.83-0.99)]. A reduced risk of colorectal cancer was not observed for the subclasses of flavonols.

The preventative effects of flavonoids on stomach and colorectal cancer risk were estimated by pooling the estimates based on the published observational studies. Total dietary flavonoid intake was not associated with a reduced risk of stomach and colorectal cancer, but several subclasses of flavonoids, mostly in case-control studies, showed protective effects against stomach and colorectal cancer risk.

Flavonoids could affect cancer risk through their anti-inflammatory and antitumor activities. Flavonoids exert their anti-inflammatory activities by inhibiting cyclooxygenase-2 (COX2) in colon cancer cells, and this is associated with a reduced risk of colorectal cancer<sup>[43]</sup>. Plant flavonoids induce apoptosis and suppress the growth of colon cancer cells by inhibiting the COX2- and Wnt/epidermal growth factor receptor/nuclear factor- $\kappa$ B-signaling pathways, which play crucial roles in colorectal cancer<sup>[44]</sup>. Quercetin inhibits tyrosine kinase activity, thus downregulating cell proliferation<sup>[45]</sup>. The antitumor effects of flavonoids have not been demonstrated con-

clusively, but it has been suggested that the free radical scavenging properties of flavonoids are closely related to beneficial effects on cancer risk, as flavonoids are more effective antioxidants than vitamin C, vitamin E and carotenoids<sup>[46]</sup>. Hydroxylation at the 3-position on the C ring, the increased number of hydroxyl groups in ring B, and a saturated 2-3 bond on the C ring showed enhanced scavenging activities<sup>[47,48]</sup>.

Reduced risks of colorectal cancer were observed in the summary estimates for flavonols, flavan-3-ols, anthocyanidins, and proanthocyanidins in the present study, as well as in subgroup analyses. The intake of total dietary flavonols and dietary quercetin were associated with a significant reduction of stomach cancer risk. These results might be attributed to the anti-inflammatory and antitumor effects of those nutrients. However, no reduced risk of stomach and colorectal cancer was observed in the summary estimates of total dietary flavonoids, including the subgroup analyses by sex and study design, in the present meta-analysis. The subclasses of flavonoids included in the calculations of total flavonoids were different across studies, as shown in Table 1. All studies included flavonols and flavones among the total dietary flavonoids, but flavan-3-ols, proanthocyanidins, which can contribute a considerable proportion of the total flavonoid intake, were not considered in most studies. These discrepancies can lead to highly heterogeneous results. However, the overall results were homogenous in terms of the effects of total flavonoids, except for the summary estimate of case-control studies, which showed moderate heterogeneity. Thus, dietary flavonoid intake might not be truly associated with stomach or colorectal cancer risk. Furthermore, dietary intake of quercetin, kaempferol, and myricetin showed no significant association with colorectal cancer risk, whereas total dietary flavonols showed statistically significant results for both cancer types. These results might be closely related to the design of the selected studies. The studies that investigated total flavonoids as well as the flavonols quercetin, kaempferol and myricetin were mostly cohort designs, while the studies that investigated total flavonols were mostly case-control designs. Case-control studies are more subject to recall bias, resulting in either an underestimate or overestimate of the risk estimates. Especially in stomach and colorectal cancer, patients might have intestinal discomfort prior to diagnosis, resulting in changes in dietary habits. Similar results were found in a meta-analysis of fruit and vegetable consumption<sup>[49]</sup>. A statistically significant association between the risk of stomach and colorectal cancer and fruit and vegetable consumption was observed only in the summary estimate of case-control studies. This summary risk estimate was highly heterogeneous, unlike that of the cohort studies, suggesting that bias might be introduced in case-control studies. Another explanation for the lack of association in cohort studies is the typically short follow-up times. In a meta-analysis of association between fruit and vegetable consumption and gastric cancer risk<sup>[50]</sup>, a



**Table 1** Selected studies on dietary flavonoids and risk of stomach and colorectal cancer

Ref.	Cancer site	Country	Study period	Case/control (n/n)	Dietary assessment method	Reported flavonoids	Included subclasses for total flavonoids	Intake comparison High vs low (mg/d) <sup>1</sup>	Controlled confounders
Garcia-Closas <i>et al</i> <sup>[20]</sup>	Stomach	Spain	1987-1989	354/354	Diet history	Q, K, M		Q4 vs Q1 mean (SD), Q: 7.1 (6.5), K: 1.2 (1.9), M: 0.65 (1.17)	Total energy intake, intake of nitrites, nitrosamines, vitamin C, total carotenoids ( $\alpha$ -carotene, $\beta$ -carotene, lutein, and lycopene) and other specific favonoids (quercetin, kaempferol, myricetin, and luteolin)
Lagiou <i>et al</i> <sup>[21]</sup>	Stomach	Greece	1981-1984	110/100	FFQ	F1, F2, F3, F4, An, I		F1: per 10.0, F2: per 0.3, F3: per 19.8, F4: per 135.1, An: per 40.4, I: per 2.0.	Age, gender, total energy intake, place of birth, BMI, height, years of education, smoking habits and duration of smoking, alcohol consumption, and fruit and vegetable consumption
Cotterchio <i>et al</i> <sup>[22]</sup>	Colorectum	Canada	1997-2000	1095/1890	FFQ	I		> 1.097 vs < 0.289.	Age, sex, and total energy intake
Rossi <i>et al</i> <sup>[23]</sup>	Colorectum	Italy	1992-1996	1953/4154	FFQ	T, F1, F2, F3, F4, An, I	F1, F2, F3, F4, An, I	T: > 191.1 vs < 75.3, F1: > 28.5 vs < 13.2, F2: > 0.7 vs < 0.3, F3: > 67.0 vs < 12.5, F4: > 88.5 vs < 20.8, An: > 31.7 vs < 5.3, I: > 33.9 vs < 14.4	Age, sex, energy intake, study center, family history of colorectal cancer, education, alcohol consumption, BMI, and occupational physical activity
Theodoratou <i>et al</i> <sup>[24]</sup>	Colorectum	Scotland	-	1456/1456	FFQ	F1, F2, F3, F4, Q, H, N, C, E, I		F1: > 36.75 vs < 16, F2: > 1.9 vs < 0.5, F3: > 45.2 vs < 16.7, F4: > 162.1 vs < 42.6, Q: > 22.9 vs < 11.7, H: > 21.1 vs < 3.95, N: > 19.7 vs < 3.8, > 62.41 vs < 24.77	Total energy intake, family history of colorectal cancer, total fiber intake, alcohol intake, NSAID intake, smoking, BMI, physical activity, and fruit and vegetable intake
Akhter <i>et al</i> <sup>[25]</sup>	Colorectum	Japan	2004-2005	721/697	FFQ	I			Age, sex, total energy intake, screening period, family history of colorectal cancer, cigarette smoking, alcohol consumption, BMI, physical activity, supplement use and non-steroidal anti-inflammatory drug use
Kyle <i>et al</i> <sup>[26]</sup>	Colorectum	United Kingdom	1998-2000	261/404	FFQ	F1, F3, F4		F1: > 40.4 vs < 19.3, F3: > 32.2 vs < 2.73, F4: > 188.8 vs < 67.1	Age, energy, family history, non-steroidal anti-inflammatory drugs, aspirin, Mn, riboflavin, vitamin C, folate
Rossi <i>et al</i> <sup>[27]</sup>	Stomach	Italy	1997-2007	230/547	FFQ	F1, F2, F3, F4, An, I, P		F1: > 32.3 vs < 13.2, F2: > 0.7 vs < 0.3, F3: > 56.8 vs < 12.9, F4: > 79.2 vs < 21.6, An: > 21.5 vs < 6.2, I: > 34.3 vs < 15.0, P: > 373.0 vs < 339.6	Age, sex, education, year of interview, BMI, tobacco smoking, and total energy intake
Rossi <i>et al</i> <sup>[28]</sup>	Colorectum	Italy	1992-1996	1953/4154	FFQ	P		> 486.6 vs < 202.5	Age, sex, study center, family history, education, alcohol consumption, BMI, physical activity and energy intake
Budhathoki <i>et al</i> <sup>[29]</sup>	Colorectum	Japan	2003-2003	816/815	FFQ	I		74.4 vs 15.5 (median)	Age, sex, total energy intake, resident area, parental colorectal cancer, smoking, alcohol use, BMI, type of job, and leisure time physical activity
Ekström <i>et al</i> <sup>[30]</sup>	Stomach (cardia and non cardia)	Sweden	1989-1995	C81, Non; 420 / 1116	FFQ	Q		> 11.89 vs < 3.88	Age, sex, socioeconomic status, number of siblings, BMI, smoking and energy and salt intake

Zamora-Ros <i>et al</i> <sup>[31]</sup>	Colorectum	Spain	1996-1998	424/401	FFQ	T, F1, F2, F3, F4, An, I, P, Q	F1, F2, F3, F4, An, I, P, Th	T: > 167.9 <i>vs</i> < 68.9, F1: > 11.5 <i>vs</i> < 5.1, F2: > 2.1 <i>vs</i> < 0.7, F3: > 17.7 <i>vs</i> < 3.7, F4: > 12.9 <i>vs</i> < 4.9, An: > 10.6 <i>vs</i> < 3.3, I: > 0.17 <i>vs</i> < 0.07, P: > 112.3 <i>vs</i> < 40.9, > 10.29 <i>vs</i> < 4.33	Age, sex, energy intake, BMI, alcohol and fiber intake, red and processed meat intake, tobacco consumption, physical activity, regular drugs, and family history of colorectal cancer
Djuric <i>et al</i> <sup>[32]</sup>	Colorectum	United States	2003-2005	1163/1501	FFQ	Q			Age, sex, physical activity at age 30-39, BMI, family history of colorectal cancer, highest education achieved, and nonsteroidal antiinflammatory drug use (NSAID) use, red meat, and total calcium intake
Follow-up (yr) Case (n)									
Hirvonen <i>et al</i> <sup>[33]</sup>	Colorectum, Stomach	Finland	6.1 (median)	C 133 S 111	Diet history	T	F1, F2	16.3 <i>vs</i> 4.2 (median)	Age and supplementation group
Knekt <i>et al</i> <sup>[34]</sup>	Colorectum, Stomach	Finland	30 (maximum)	C 90 S 74	Diet history	T, Q, K, M, H, N	F1, F2, F3	T: > 39.5 (F), 26.9 (M) <i>vs</i> < 8.5 (F), 4.3 (M), Q: > 4.7 (F), 3.9 (M) <i>vs</i> < 1.8 (F), 1.5 (M), K: > 0.9 (F), 0.8 (M) <i>vs</i> < 0.2 (F), 0.1 (M), M: > 0.2 (F), 0.11 (M) <i>vs</i> < 0.03 (F), 0.06 (M), H: > 26.8 (F), 15.4 (M) <i>vs</i> < 3.2 (F), 0 (M), N: > 7.7 (F), 4.7 (M) <i>vs</i> < 0.9 (F), 0 (M)	Age, sex, geographic area, occupation, smoking, and BMI
Lin <i>et al</i> <sup>[35]</sup>	Colorectum	United States	-	878	FFQ	T, Q, K, M	F1, F2	Q5 <i>vs</i> Q1 (NHS: > 31.1 <i>vs</i> < 9.6, HPFS: > 30.5 <i>vs</i> < 10.7)	Age, BMI, family history of colorectal cancer, history of colorectal polyps, prior sigmoidoscopy screening, physical activity, smoking status, red meat intake, alcohol consumption, total energy intake, total calcium intake, total folate intake, total fiber intake, aspirin use, and multivitamin use
Akhter <i>et al</i> <sup>[36]</sup>	Colorectum	Japan	7.6 (mean)	886	FFQ	I		Q4 <i>vs</i> Q1	Age, public health center area, history of diabetes mellitus, BMI, leisure time physical activity, cigarette smoking, alcohol drinking, and intake of vitamin D, dairy products, meat, fruit, vegetable, and fish, (F) + menopausal status and current use of female hormones
Mursu <i>et al</i> <sup>[37]</sup>	Colorectum	Finland	16.2 (mean)	55	Food recording	T, F1, F2, F3, F4, An	F1, F2, F3, F4, An	Q4 <i>vs</i> Q1 T: 416.3 <i>vs</i> 265.0 (mean)	Age and examination years, BMI, smoking status, pack-years of smoking, physical activity, intakes of alcohol, total fat and saturated fat, and energy adjusted intake of fiber, vitamin C and E
Simon <i>et al</i> <sup>[38]</sup>	Colorectum	Netherlands	13.3	1271	FFQ	T, F4	F1, F2	T: > 36 <i>vs</i> < 16 (M), F4: > 84.3 <i>vs</i> < 44.4 (M), T: > 38.3 <i>vs</i> < 18.4 (F), F4: > 95.9 <i>vs</i> < 51.6 (F)	Age, family history of colorectal cancer, smoking status, alcohol intake, occupational physical activity at longest held job, BMI and processed meat intake
Wang <i>et al</i> <sup>[39]</sup>	Colorectum	United States	11.5 (mean)	305	FFQ	T	F1, F2	T: > 34.6 <i>vs</i> < 11.6	Age, race, total energy intake, and randomized treatment assignment, smoking, alcohol use, physical activity, postmenopausal status, hormone replacement therapy use, multivitamin use, BMI, family history of colorectal cancer, ovary cancer, and breast cancer, and intake of fruit and vegetables, fiber, folate, and saturated fat
Yang <i>et al</i> <sup>[40]</sup>	Colorectum	United States	6.4 (mean)	321	FFQ	I		T3 <i>vs</i> T1 34.8 <i>vs</i> 20.9 (mean)	Age, education, household income, BMI, physical activity, menopausal status, family history of colorectal cancer, total calorie intake, and average intakes of fruit, vegetables, red meat, nonsoy calcium, nonsoy fiber, and nonsoy folic acid

Ward <i>et al</i> <sup>[41]</sup>	Colorectum	United States	9 (mean) 221	Food recording	I	Continuous	Age, height, weight, family history of colorectal cancer, smoking status, aspirin use, physical activity, and average daily intake of fat, energy, calcium, alcohol, and red and processed meats, (F) + oral contraceptive use, menopausal status, menopausal hormone therapy use, parity, breastfeeding, and surgical removal of ovaries
Hara <i>et al</i> <sup>[42]</sup>	Stomach	Japan	1249	FFQ	I	Q4 vs Q1 (median) 42.3 vs 9.2 (M) 41.8 vs 9.4 (F)	Age, public center area, BMI, smoking status, ethanol intake, family history of gastric cancer, vegetable, fruit, fish, salt, and total energy intake

<sup>1</sup>M and F represent male and female in this row. Q: Quercetin; K: Kaempferol; M: Myricetin; H: Hesperidin; N: Naringenin; C: Catechin; E: Epicatechin; F1: Flavonols; F2: Flavones; F3: Flavanones; F4: Flavan-3-ols; An: Anthocyanidins; I: Isoflavones; P: Proanthocyanidins; Th: Theaflavins; BMI: Body mass index.

**Table 2 Summary estimates of dietary flavonoids and stomach and colorectal cancer risk**

	Combined						Colorectum					Stomach				
	<i>n</i> <sup>a</sup>	RR	95%CI	Heterogeneity		<i>n</i>	RR	95%CI	Heterogeneity		<i>n</i>	RR	95%CI	Heterogeneity		<i>P</i> <sup>b</sup>
				<i>I</i> <sup>2</sup>	<i>P</i> <sup>b</sup>				<i>I</i> <sup>2</sup>	<i>P</i> <sup>b</sup>				<i>I</i> <sup>2</sup>	<i>P</i> <sup>b</sup>	
Total flavonoids	11	1.00	0.91-1.11	15.0%	0.301	9	1.00	0.90-1.11	28.2%	0.194	2	1.07	0.70-1.61	0%	0.464	
Cohort	9	1.05	0.93-1.18	0%	0.517	7	1.04	0.92-1.18	9.6%	0.355	2	-	-	-	-	
Case-control	-	-	-	-	-	2	0.81	0.50-1.29	67.9%	0.078 <sup>c</sup>	0	-	-	-	-	
Female	-	-	-	-	-	3	0.93	0.84-1.04	0%	0.890	0	-	-	-	-	
Male	5	1.05	0.96-1.15	0%	0.514	4	1.05	0.96-1.15	18.6%	0.298	1	-	-	-	-	
Flavonols	7	0.71	0.63-0.80	10.7%	0.348	5	0.71	0.63-0.81	37.0%	0.175	2	0.68	0.46-0.99	0%	0.586	
Case-control	6	0.70	0.62-0.79	0%	0.755	4	0.70	0.62-0.79	0%	0.510	2	-	-	-	-	
Female	-	-	-	-	-	2	0.84	0.75-0.93	0%	0.361	0	-	-	-	-	
Male	-	-	-	-	-	3	0.87	0.79-0.96	45.4%	0.160	0	-	-	-	-	
Quercetin	8	0.81	0.68-0.97	44.1%	0.085 <sup>c</sup>	4	0.90	0.80-1.03	42.2%	0.159	4	0.66	0.51-0.85	0%	0.472	
Kaempferol	4	0.95	0.65-1.37	55.2%	0.082 <sup>c</sup>	2	1.12	0.91-1.38	0%	0.979	2	0.73	0.31-1.71	71.8%	0.060 <sup>c</sup>	
Myricetin	4	1.15	0.87-1.51	0%	0.965	2	1.15	0.80-1.67	0%	0.607	2	1.13	0.75-1.71	0%	0.935	
Flavones	6	0.84	0.74-0.96	44.8%	0.107	4	0.83	0.63-1.09	64.4%	0.038 <sup>c</sup>	2	0.76	0.54-1.08	0%	0.635	
Case-control	5	0.82	0.66-1.02	55.0%	0.064 <sup>c</sup>	3	0.83	0.61-1.14	87.9%	0.016 <sup>c</sup>	2	-	-	-	-	
Female	-	-	-	-	-	2	0.94	0.84-1.04	0%	0.353	0	-	-	-	-	
Male	-	-	-	-	-	3	0.92	0.84-1.02	0%	0.477	0	-	-	-	-	
Flavanones	7	1.04	0.86-1.27	44.5%	0.094 <sup>c</sup>	5	1.08	0.95-1.22	28.2%	0.234	2	0.72	0.49-1.07	38.3%	0.203	
Case-control	6	1.05	0.85-1.30	53.3%	0.057 <sup>c</sup>	4	1.08	0.95-1.23	46.4%	0.144	2	-	-	-	-	
Female	-	-	-	-	-	2	1.01	0.89-1.14	0%	0.734	0	-	-	-	-	
Male	-	-	-	-	-	3	0.99	0.89-1.11	0%	0.583	0	-	-	-	-	
Hesperidin	3	1.12	0.90-1.40	0%	0.675	2	1.15	0.91-1.45	0%	0.587	1	-	-	-	-	
Naringenin	3	1.11	0.89-1.38	0%	0.732	2	1.13	0.90-1.42	0%	0.539	1	-	-	-	-	
Flavan-3-ols	9	0.88	0.80-0.97	16.3%	0.297	7	0.88	0.79-0.97	30.1%	0.198	2	0.91	0.66-1.26	0%	0.335	
Cohort	-	-	-	-	-	3	0.91	0.76-1.08	26.9%	0.254	0	-	-	-	-	
Case-control	6	0.87	0.77-0.98	24.7%	0.249	4	0.86	0.76-0.98	46.6%	0.132	2	-	-	-	-	
Female	-	-	-	-	-	3	0.94	0.87-1.02	54.7%	0.110	0	-	-	-	-	
Male	-	-	-	-	-	4	1.07	0.99-1.15	23.5%	0.270	0	-	-	-	-	
Anthocyanidines	5	0.75	0.64-0.89	21.9%	0.275	3	0.68	0.56-0.82	0%	0.868	2	1.03	0.73-1.43	0%	0.509	
Case-control	4	0.76	0.64-0.90	37.9%	0.185	2	0.68	0.56-0.83	0%	0.668	2	-	-	-	-	
Proanthocyanidines	3	0.55	0.35-0.87	74.4%	0.020 <sup>c</sup>	2	0.72	0.61-0.85	0%	0.372	1	-	-	-	-	
Isoflavones	14	0.90	0.80-1.01	54.4%	0.008 <sup>c</sup>	10	0.89	0.77-1.02	65.1%	0.003 <sup>c</sup>	4	0.91	0.78-1.06	5.5%	0.206	
Cohort	7	0.98	0.85-1.12	44.0%	0.098	5	1.02	0.90-1.15	43.1%	0.135	2	0.88	0.74-1.05	48.7%	0.163	
Case-control	7	0.78	0.70-0.87	37.5%	0.143	5	0.76	0.68-0.85	36.2%	0.180	2	1.02	0.73-1.44	0%	0.430	
Female	7	0.95	0.81-1.11	47.7%	0.075 <sup>c</sup>	6	0.93	0.78-1.11	54.3%	0.052 <sup>c</sup>	1	-	-	-	-	
Male	6	0.89	0.82-0.96	35.7%	0.170	5	0.90	0.83-0.99	42.0%	0.141	1	-	-	-	-	

<sup>a</sup>Selected study numbers; <sup>b</sup>*P* values for heterogeneity from Q-test; <sup>c</sup>Random effects model was used if *P* < 0.1. RR: Relative risk.

significant association was observed only in the meta-analyses of studies with more than 10 years of follow-up. Meta-regression also revealed that longer follow-up time was associated with lower risk of gastric cancer. The mean follow-up times were more than 10 years for all cohort studies reporting total dietary flavonoid intake in our study, except for Hirvonen *et al*<sup>[33]</sup>. A meta-analysis performed after excluding this study did not change the results. Thus, the hypothesis of an inverse association

between dietary flavonoids and risk of stomach and colorectal cancer cannot be supported by our results.

Although this meta-analysis provided little evidence of an inverse association between stomach and colorectal cancer risk and flavonoid intake, several mechanisms supported by *in vitro* and animal studies remain biologically plausible. Antitumor effects were shown for quercetin, luteolin, and myricetin<sup>[51-54]</sup>, and polyphenols extracted from apple and olive oil had a chemopreven-

tive effect in colon cancer cell lines or mice<sup>[55-57]</sup> and antioxidant activities in gastric mucosa<sup>[58,59]</sup>. Most subclasses of flavonoids were inversely associated in the present meta-analyses, although some bias might have been introduced. Thus, it is thought that dietary flavonoids are inversely associated with stomach or colorectal cancer risk, but this association is very small, making it difficult to detect. The United States Department of Agriculture (USDA) database for food flavonoids has been updated several times since 2003<sup>[60]</sup>. Most total dietary flavonoids are flavan-3-ols (82.5%), as estimated by USDA database and 24 h dietary records provided by United States adults<sup>[61]</sup>. The second greatest contributor was flavanones (7.6%), followed by flavonols (6.8%), anthocyanidins (1.6%), flavones (0.8%), and isoflavones (0.6%). However, the bioavailability of these compounds might be different. For example, tea flavonoids showed low absorption rates, whereas quercetin and isoflavones showed strong bioavailability. The main source of dietary flavonoid subclasses in Scotland in a database by Kyle *et al.*<sup>[62]</sup> was tea<sup>[24]</sup>, whereas the main sources of total flavonoids in Spain<sup>[31]</sup>, calculated using the USDA database<sup>[60]</sup>, were fruits (65.1%), followed by wine (14.4%), legumes (6.3%), and vegetables (4.2%). The methods used to estimate total flavonoids from dietary intake are poorly established<sup>[61]</sup>. Levels of exact individual flavonoid intake from food should be determined to confirm the true association between flavonoids and stomach and colorectal cancer risk.

The main limitation of this study is the small number of publications included. Especially in the case of stomach cancer, summary estimates could not be calculated in several subgroup analyses due to the limited number of studies. Because a significant association was found in the analyses of case-control studies which were subject to bias, the inverse association between subclasses of dietary flavonoid intake and cancer risk may be overestimated.

## CONCLUSION

There was no clear evidence that dietary flavonoids are associated with reduced risk of stomach and colorectal cancer in the present meta-analysis. However, there is a possibility that there may be a weak association with stomach and colorectal cancer based on consistent associations for subclasses of flavonoids. The association of flavonoids with stomach and colorectal cancer could be relatively small and thus might only be detected with better methods of estimating true dietary flavonoid intake.

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## Consumption of red and processed meat and esophageal cancer risk: Meta-analysis

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### Abstract

To summarize the evidence about the association between red and processed meat intake and the risk of esophageal cancer, we systematically searched the PubMed and EMBASE databases up to May 2012, with a restriction to English publications, and the references of the retrieved articles. We combined the study-specific relative risks (RRs) and 95%CI, comparing the highest with the lowest categories of consumption by using a random-effects model. A total of 4 cohort studies and 23 case-control studies were included in the meta-analysis. The combined RRs (95%CI) of the cohort studies comparing the highest and lowest categories were 1.26 (1.00-1.59) for red meat and 1.25 (0.83-1.86) for processed meat. For the case-control studies, the combined RRs (95%CI) comparing the highest and lowest categories were 1.44 (1.16-1.80)

for red meat and 1.36 (1.07-1.74) for processed meat. Findings from this meta-analysis suggest that a higher consumption of red meat was associated with a greater risk of esophageal cancer.

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**Key words:** Cohort study; Case-control study; Meta-analysis; Red meat; Processed meat; Esophageal cancer

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### INTRODUCTION

The incidence rate of esophageal cancer ranked eighth worldwide, accounting for 3.8% of all new cancers, and its mortality rate ranked sixth, accounting for 5.4% of all cancer deaths in 2008<sup>[1]</sup>. The most predominant histological types of esophageal cancer are esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC), representing distinct characteristics in patterns of cancer development and risk factors<sup>[2]</sup>.

Given that mutagenic compounds such as heterocyclic amines (HCAs), polycyclic aromatic hydrocarbons (PAHs), and N-nitroso compounds (NOCs) generated from red and processed meats were associated with cancer development<sup>[3]</sup>, concerns about a high incidence of esophageal cancer related to a high consumption of red and processed meats have been increasing. In 2007, a consensus report of experts assembled by the World Cancer Research Fund (WCRF) and the American Institute for Cancer Research (AICR)<sup>[4]</sup> concluded through review of studies published up to 2004 that there were suggestive but inconclusive associations between red and processed meat consumption

and esophageal cancer risk. The WCRF/AICR expert report also indicated that the lack of consistent results may be because of insufficient data, especially from prospective cohort studies. Another review of studies published up to 2005<sup>[5]</sup> suggested a possible increased risk of esophageal cancer with processed meat (9 case-control studies) and combined white and red meat (2 cohort and 18 case-control studies); however, this study concluded that more prospective data involving a larger number of cases would be needed to determine the association between meat consumption and the risk of esophageal cancer.

Since the completion of the two reviews, the results of the large prospective studies as well as new or updated case-control studies that examined association between red and processed meats and esophageal cancer risk have been published, but no meta-analysis of the prospective cohort studies has been reported. We, therefore, performed a meta-analysis of large prospective cohort and case-control studies to summarize the association between red and processed meat intake and the risk of esophageal cancer. We also quantified the dose-response relationships in the analysis of the cohort studies.

## SEARCH STRATEGY

Two authors (Choi Y, Song S) independently performed a systematic search of published articles using the PubMed and EMBASE databases up to May 2012<sup>[6]</sup>. We used the following search terms: “oesophageal or esophageal or esophagus or oesophagus” and “cancer or neoplasm or carcinoma” and “cohort or prospective or case-control” and “food or diet or meat”. We also reviewed the reference lists from the retrieved articles and those from previous review studies to identify additional relevant studies that may not have been identified by our database searches.

## INCLUSION CRITERIA

Studies were included in our meta-analysis if they met the following criteria: (1) either a cohort or case-control design was used; (2) relative risk (RR) estimates and the 95%CI were provided for the association between red and/or processed meat intake and esophageal cancer; (3) the outcomes of interest were either the overall incidence of esophageal cancer or the two main histological subtypes, ESCC or EAC; and (4) the study was published in English. We included studies that reported the associations of esophageal cancer with exposures identified as “red meat” or “processed meat” and individual food items within the two groups. Studies generally included beef, pork, minced meat, lamb, veal, and offal (*e.g.*, liver, kidney) for unprocessed red meat and sausage, ham, bacon, salami, luncheon meat, or frankfurters, and any types of meat that were processed by smoking, curing, salting, or the addition of preservatives for processed meat. We excluded studies providing no apparent classification of meat or studies reporting a combination of red and white meat (*e.g.*, poultry). If data were duplicated in more than 1 study, the latest studies were included.

## DATA EXTRACTION

We independently extracted the following data from each study, according to the meta-analysis of observational studies in epidemiology guidelines<sup>[6]</sup>, and any discrepancies were resolved by discussion: the first author's last name, the publication year, the country where the study was conducted, the study period, the age range of the subjects, the number of cases and controls or the cohort size, the measures and comparison levels of the exposures, the multivariate adjusted RRs with corresponding 95%CI for the highest vs lowest categories of red or processed meat intake, and the variables that were adjusted for in the analysis. For each study, we used the most fully adjusted RRs in the multivariate model. Any disagreements were resolved through consensus. The same two authors assessed the quality of the studies based on the Newcastle-Ottawa Scale, which ranged from 1 to 9 stars<sup>[7]</sup>. The average score for each study was used in the analysis.

## STATISTICAL ANALYSIS

We conducted separate meta-analyses for case-control and cohort studies, using results that compared red and processed meat intake as well as those that assessed each type individually. We also performed a meta-analysis combining both case-control and cohort studies. Using a random-effects model that considered both within and between study variation<sup>[8]</sup>, we combined the study-specific multivariate RRs and 95%CI, comparing the highest and the lowest categories of red and processed meat intake.

We assessed the statistical heterogeneity among the studies by using  $Q$  and  $I^2$  statistics<sup>[9]</sup>, where significance was reached at  $P < 0.1$ . Publication bias was evaluated by using the Egger asymmetry test<sup>[10]</sup>, with significant level at  $P < 0.05$ . We investigated the potential sources of heterogeneity among the studies by conducting subgroup and meta-regression analyses for histological subtype (ESCC and EAC), sex (males, females, and both sexes), study location (Asia, Europe, North America, and South America), study quality, and confounders adjusted for in the analysis [alcohol, smoking, body mass index (BMI), and fruit and/or vegetable]. We also conducted the sensitivity analysis for case-control and cohort studies separately, omitting each study individually to evaluate whether the results could have been affected substantially by any one study.

In a sensitivity analysis, we estimated a dose-response for combined RRs for 100 g/d increments of red or processed meats for 3 cohort studies<sup>[11-13]</sup>, which are less prone to selection or recall bias than case-control studies. We did not include one study (Yu *et al.*<sup>[14]</sup>), that presented binary categories of exposure for a dose-response analysis. For two studies<sup>[11,13]</sup>, the estimates were rescaled into 100 g/d increments. All statistical analyses were performed with Stata software, version 11 (Stata Corp., College Station, TX, United States).  $P < 0.05$  considered statistically significant.



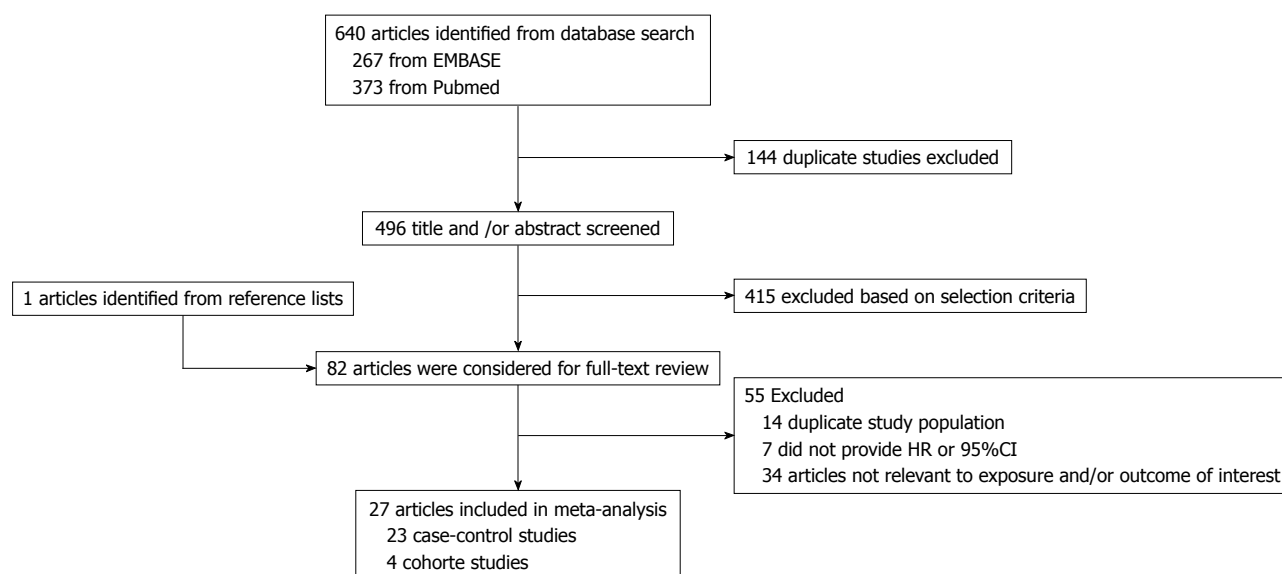


Figure 1 Selection of studies included in meta-analysis.

## LITERATURE SEARCH

The preliminary literature search yielded 640 articles. Of these, 81 articles and 1 additional article identified from the reference lists were considered for further review (Figure 1). After the full-text review, 7 articles that did not provide RRs or 95%CI, 14 articles that used duplicated study populations, and 34 articles that were unrelated to exposure or outcomes of interest were excluded. A total of 27 articles were included in the meta-analysis; 22 articles (4 cohort and 18 case-control studies) that reported findings on red meat and 18 articles (3 cohorts and 15 case-controls) that reported findings on processed meat were included in the meta-analysis.

## RED MEAT INTAKE

We identified 4 cohort studies<sup>[11-14]</sup> involving 2324 cases and 1 149 981 participants and 18 case-control studies<sup>[15-32]</sup> involving 5165 cases and 26 350 control subjects (Table 1). Two of the 22 studies reported results for both ESCC and EAC, 16 studies reported the results for either EAC or ESCC, and 6 reported results for overall esophageal cancer without the histological subtypes. Six studies were conducted in Asia, 6 in Europe, 7 in United States, and 3 in South America. The studies used either a food frequency questionnaire (FFQ) or a structured questionnaire form to measure red meat intake. Fifteen studies provided RR estimates that were adjusted for alcohol intake, 16 for smoking habit, 12 for BMI, and 7 for fruit and/or vegetable intake. Eight studies were given a score of 7 stars or above, representing a high quality of studies<sup>[7]</sup>. The combined RRs (95%CI) comparing the highest and lowest categories of red meat intake were 1.26 (1.00-1.59) for the 4 cohort studies and 1.44 (1.16-1.80) for the 18 case-control studies (Figure 2A). There was no evidence of heterogeneity among the cohort stud-

ies ( $P = 0.15$ ,  $I^2 = 35.3\%$ ), but there was a heterogeneity among the case-control studies ( $P < 0.01$ ,  $I^2 = 72.8\%$ ). Combining the two types of study design resulted in an overall combined RR of 1.38 (95%CI: 1.17-1.64;  $P$  for heterogeneity:  $P < 0.01$ ,  $I^2 = 67.1\%$ ). Excluding a single study did not substantially influence the combined estimates of the cohort or case-control studies. There was no statistical evidence of publication bias according to the Egger asymmetry test ( $P = 0.79$  for cohort studies and  $P = 0.34$  for case-control studies). Dose-response associations were examined in 3<sup>[11-13]</sup> of 4 cohort studies, showing the combined RRs of 1.05 (95%CI: 0.91-1.21;  $P$  for heterogeneity = 0.42,  $I^2 = 0.2\%$ ) for every 100 g/d increment of red meat intake. The associations did not vary significantly by histological subtypes, study location, sex, and study quality (Table 2). In addition, the associations did not differ by adjusted confounding factors including alcohol, smoking, BMI, and fruit and vegetable intakes (data not shown).

## PROCESSED MEAT INTAKE

We conducted a meta-analysis of 3 cohort studies<sup>[11-13]</sup>, which included 1162 cases and 1 137 288 participants and 15 case-control studies<sup>[15,16,19-21,24,25,27,30,32-37]</sup>, which included 3851 cases and 10 064 controls (Table 1). Two of the 18 studies examined both ESCC and EAC as the primary endpoints, 13 studies reported the results for either EAC or ESCC and 5 did not differentiate between histological subtypes. Five studies were conducted in Asia, 7 in Europe, 5 in United States, and 1 in South America. The studies used either a FFQ or a structured questionnaire form to measure processed meat intake. Fourteen studies provided RR estimates that were adjusted for alcohol intake, 15 for smoking habit, 10 for BMI, and 8 for fruit and/or vegetable intake. Six studies were given a score of 7 or greater, indicating a high methodological quality<sup>[7]</sup>.

Table 1 Characteristics of the studies included in the meta analysis

Ref.	Study period	Sex	No. of cases	No. cohorts or controls	Dietary assessment	Exposure and comparison level	Adjusted RR (95%CI)	Study quality <sup>1</sup>	Adjustment for confounders							
Cohort studies																
Keszei <i>et al</i> <sup>[11]</sup>	1986-2002	M	ESCC: 107	120 852	FFQ 150 items	Red meat		9	Age, smoking (including years and numbers per day), total energy, BMI, alcohol drinking, vegetable, fruit, education, non-occupational PA							
		F	EAC: 145			ESCC										
		M				Q5 <i>vs</i> Q1	2.66 (0.94-7.48)									
		F				T3 <i>vs</i> T1	0.87 (0.42-1.79)									
						EAC										
		M				Q5 <i>vs</i> Q1	0.57 (0.28-1.19)									
		F				T3 <i>vs</i> T1	1.09 (0.44-2.75)									
						Processed meat										
						ESCC										
		M				Q5 <i>vs</i> Q1	3.47 (1.21-9.94)									
Cross <i>et al</i> <sup>[12]</sup>	1995-2006	C	ESCC: 215	494 979	FFQ 124 items	Red meat (Q5 <i>vs</i> Q1)		9	Age, sex, BMI, education, ethnicity, smoking, alcohol drinking, PA at work, vigorous PA, daily intakes of fruit, vegetable, saturated fat, energy							
			EAC: 630			ESCC	1.79 (1.07-3.01)									
						EAC	1.15 (0.84-1.57)									
						Processed meat (Q5 <i>vs</i> Q1)										
						ESCC	1.32 (0.83-2.10)									
						EAC	1.08 (0.81-1.43)									
		González <i>et al</i> <sup>[13]</sup>	1992-1998			C	EAC: 65			521 457	FFQ 88-266 items	Red meat (T3 <i>vs</i> T1)	1.67 (0.75-3.72)	8	Sex, height, weight, education, smoking, smoking intensity, work and leisure PA, intakes of alcohol, energy, vegetable, citrus fruit, non-citrus fruit, types of meat intake were mutually adjusted	
												Processed meat (T3 <i>vs</i> T1)	3.54 (1.57-7.99)			
Yu <i>et al</i> <sup>[14]</sup>	1974-1989	C	All: 1162	12 693	Questionnaire 15 items	Pork (never <i>vs</i> regular/occasional)	1.37 (1.11-1.68)	7	Age, sex							
Case-control studies																
Ward <i>et al</i> <sup>[15]</sup>	1988-1993	C	EAC: 124	449	Questionnaire 100 items	Red meat (> 157.2 g/d <i>vs</i> ≤ 73.8 g/d)	2.85 (1.00-8.16)	5	Age, sex, race, vital status, year of birth, sex, No. of cigarettes per day, BMI, intakes of retinoic acid, folate, riboflavin, zinc, carbohydrate, protein, total energy.							
						Processed meat (> 52.3 g/d <i>vs</i> ≤ 16.1 g/d)	1.40 (0.62-3.15)									
De Stefani <i>et al</i> <sup>[16]</sup>	1996-2004	C	ESCC: 234	2020	FFQ 64 items	Red meat (T3 <i>vs</i> T1)	4.97 (2.98-8.29)	7	Age, sex, residence, education, BMI, smoking, drinking, mate temperature, total energy, total intakes of vegetable and fruit, scored pattern							
						Processed meat (T3 <i>vs</i> T1)	0.76 (0.51-1.13)									
Gao <i>et al</i> <sup>[17]</sup>	1997-2005	C	ESCC: 600	1514	Questionnaire 35 items	Red meat (> weekly <i>vs</i> monthly/seldom/never)	1.37 (1.03-1.82)	5	Age, sex, geographic region							
Wu <i>et al</i> <sup>[18]</sup>	2003-2007	C	All: 1495	3819	FFQ	Red meat (Q4 <i>vs</i> Q1)	1.13 (0.94-1.36)	7	Age, sex, education, previous income, BMI, pack-years smoking, weekly ethanol intake, study area							
Hajizadeh <i>et al</i> <sup>[19]</sup>	N/A	C	ESCC: 47	96	FFQ 168 items	Red meat (T3 <i>vs</i> T1)	2.47 (0.76-7.96)	6	Age, sex, education, tobacco smoking, symptomatic gastroesophageal reflux, BMI, total energy							
						Processed meat (T3 <i>vs</i> T1)	1.10 (0.36-2.47)									
O'Doherty <i>et al</i> <sup>[20]</sup>	2002-2005	C	EAC: 221	256	FFQ 101 items	Red meat (Q4 <i>vs</i> Q1)	3.15 (1.38-7.20)	7	Age, sex, smoking, BMI 5 yr before interview date, education, job type, Intakes of energy, fruit, vegetable, alcohol (g/d), <i>Helicobacter pylori</i> infection, nonsteroidal anti-inflammatory drug use 5 yr before, interview date, gastroesophageal reflux symptoms, location, types of meat intake were mutually adjusted							
						Processed meat (Q4 <i>vs</i> Q1)	1.41 (0.67-2.95)									

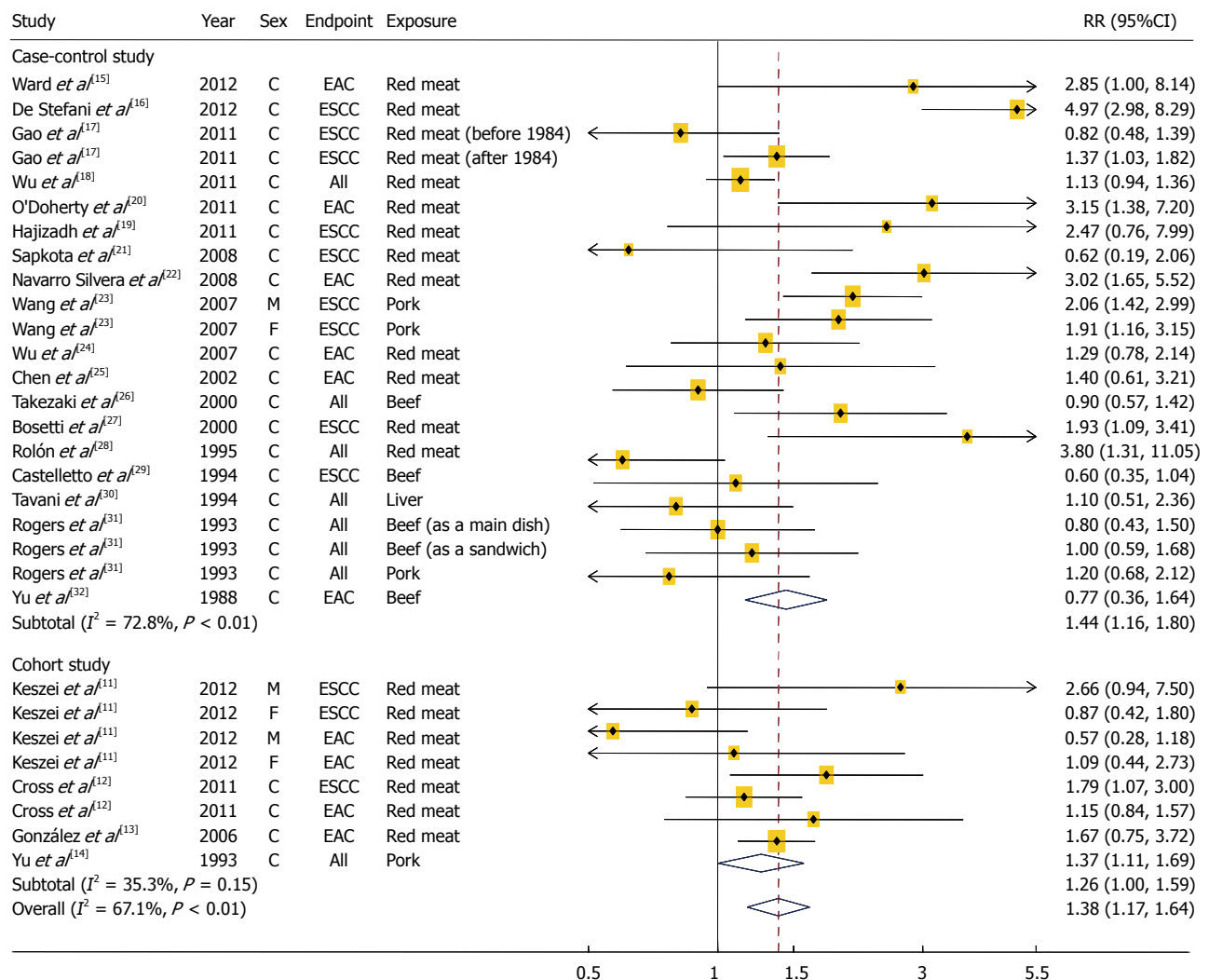
Sapkota <i>et al</i> <sup>[21]</sup>	1999-2003	C	ESCC: 187	1110	Questionnaire 23 items	Red meat ( $\geq 1/\text{wk}$ vs $< 1/\text{mo}$ ) Processed meat ( $\geq 1$ time/wk vs $< 1$ time/mo)	0.62 (0.19-2.09) 1.12 (0.52-2.41)	6	Age, sex, country, tobacco pack-year, education, BMI, frequency of alcohol consumption, vegetable, fruit consumption
Navarro Silvera <i>et al</i> <sup>[22]</sup>	1993-1995	C	EAC: 282	687	FFQ 104 items	Red meat (high vs low)	3.02 (1.65-5.52)	7	Age, sex, study site, race, proxy status, income, education, BMI, No. of smoking cigarettes per day, intakes of beer, wine, liquor, and energy
Wang <i>et al</i> <sup>[23]</sup>	2004-2006	M	ESCC: 355	408	Questionnaire	Pork (often vs none/seldom)	2.06 (1.42-2.99) 1.91 (1.16-3.16)	5	Age, sex, marital status, education
Wu <i>et al</i> <sup>[24]</sup>	1992-1997	C	EAC: 206	1308	Questionnaire 124 items	Red meat (Q4 vs Q1) Processed meat (Q4 vs Q1)	1.29 (0.8-2.2) 1.23 (0.7-2.1)	5	Age, sex, race, birthplace, education, smoking, BMI, reflux, use of vitamins, total energy
Chen <i>et al</i> <sup>[25]</sup>	1988-1993	C	EAC: 124	449	Questionnaire 54 items	Red meat (Q4 vs Q1) Processed meat (Q4 vs Q1)	1.4 (0.61-3.2) 1.7 (0.71-3.9)	7	Age, sex, energy intake, respondent type, BMI, alcohol drinking, smoking, education, family history, vitamin supplement use, age squared for EAC
Takezaki <i>et al</i> <sup>[26]</sup>	1988-1997	M	All: 284	11 888	Questionnaire	Beef ( $\geq 3/\text{wk}$ vs $\leq 3/\text{mo}$ )	0.9 (0.6-1.5)	5	Age, year and season of visit, smoking, drinking
Bosetti <i>et al</i> <sup>[27]</sup>	1992-1997	C	ESCC:304	743	FFQ 78 items	Red meat (Q5 vs Q1) Processed meat (Q5 vs Q1)	1.93 (1.09-3.41) 1.39 (0.85-2.26)	5	Age, sex, area of residence, education, tobacco smoking, alcohol drinking, non-alcohol energy
Rolón <i>et al</i> <sup>[28]</sup>	1988-1991	C	All: 131	379	FFQ	Red meat (highest vs lowest)	3.8 (1.3-11.0)	5	Age, sex, alcohol, smoking, design variable of the study, hospital group, intakes of red meats, fats, fish, milk
Castelletto <i>et al</i> <sup>[29]</sup>	1986-1989	C	ESCC: 131	261	FFQ 10 food groups	Beef ( $\geq$ daily vs $<$ daily)	0.6 (0.3-0.9)	6	Age, sex, design variable, hospital, education, No. of cigarettes smoking per day, intakes of alcohol, barbecued meat, potatoes, raw vegetables, cooked vegetables
Tavani <i>et al</i> <sup>[30]</sup>	1984-1992	C	All: 46	230	FFQ 14 items	Ham (Q3 vs Q1) Liver (Q2 vs Q1)	1.4 (0.6-3.3) 1.1 (0.5-2.3)	5.5	Age, sex, education, total alcohol intake
Rogers <i>et al</i> <sup>[31]</sup>	1983-1987	C	All: 127	466	FFQ 125 items	Beef ( $\geq 1/\text{wk}$ vs $< 1/\text{wk}$ ) As a main dish As a sandwich Pork ( $\geq 1/\text{wk}$ vs $< 1/\text{wk}$ )	0.8 (0.4-1.4) 1.0 (0.6-1.7) 1.2 (0.8-2.5)	5	Age, sex, pack-years of cigarette, drink-years of alcohol, energy intake, beta-carotene intake, ascorbic acid intake
Yu <i>et al</i> <sup>[32]</sup>	1975-1981	C	Beef: 267 Fried bacon or ham: 265 Barbecued or smoked meat: 268	Beef: 267 Fried bacon or ham: 265 Barbecued or smoked meat: 268	Questionnaire 10 food groups	Beef ( $\geq 5/\text{wk}$ vs $\leq 1/\text{wk}$ ) Fried bacon or ham ( $\leq 1/\text{wk}$ vs $\geq 5/\text{wk}$ ) Barbecued or smoked meat ( $\geq 2/\text{wk}$ vs $\leq 1/\text{wk}$ )	1.3 (0.6-2.7) 2.0 (1.1-3.5) 1.7 (0.9-3.0)	5	Age, sex, race
Chen <i>et al</i> <sup>[33]</sup>	1996-2005	M	ESCC: 320	709	Questionnaire 6 items	Cured meat ( $\geq 1/\text{wk}$ vs $< 1/\text{wk}$ )	0.8 (0.4-1.4)	5	Age, educational level, ethnicity, source of hospital, smoking, alcohol drinking, areca nut chewing
Yang <i>et al</i> <sup>[34]</sup>	2003-2004	C	All: 185	185	Questionnaire 9 Items	Processed meat ( $> 3$ meals/wk vs $< 1$ meal/wk)	0.66 (0.31-1.41)	5.5	Family history of esophageal cancer, occupation, smoking, drinking, eating hot food, eating speed, intakes of vegetables, fruit, pickled vegetables, fresh meat, egg, tea, water supply
Levi <i>et al</i> <sup>[35]</sup>	1992-2002	C	All:138	660	FFQ 79 items	Processed meat ( $> 3.2$ freq/wk vs $< 0.8$ freq/wk)	4.48 (2.05-9.79)	6	Age, sex, education, smoking, intakes of alcohol, energy, fruit and vegetable intake
Li <i>et al</i> <sup>[36]</sup>	1997-2000	C	All:1248	1248	Questionnaire 12 items	Sowbelly (daily vs $< 1/\text{wk}$ )	2.28 (1.6-3.3)	5	Age, sex, income, residence, occupation, alcohol, tobacco

<sup>1</sup>Study quality was assessed using the Newcastle-Ottawa Scale (range: 1-9 stars). RR: Relative risk; M: Male; F: Female; C: Combined males and females; ESCC: Esophageal squamous cell carcinoma; EAC: Esophageal adenocarcinoma; FFQ: Food frequency questionnaire; BMI: Body mass index; PA: Physical activity; N/A: Not available.

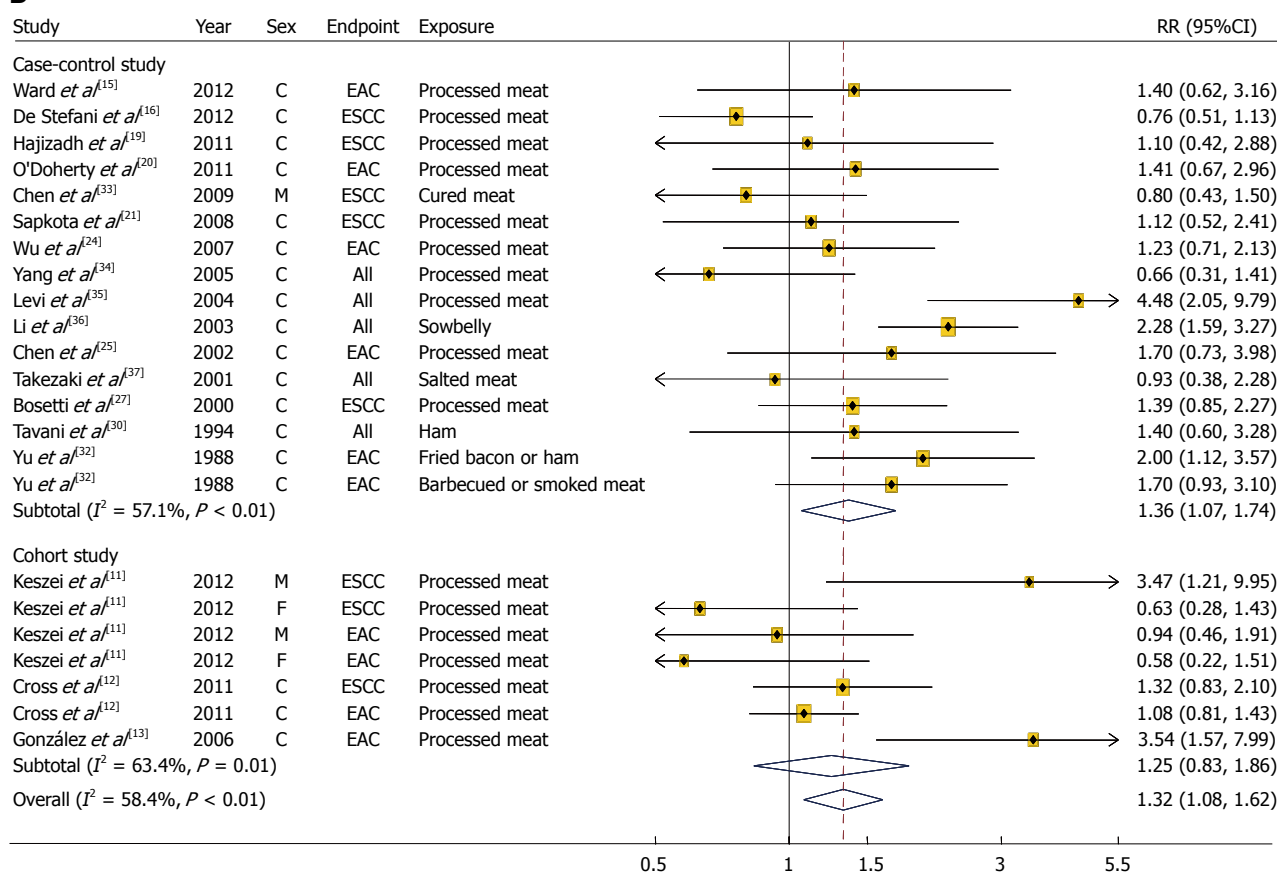
**Table 2** Combined relative risks and 95%CI for esophageal cancer associated with red meat or processed meat by other factors in both cohort and case-control studies

Factors	Red meat				Processed meat			
	Studies	Ref.	RR (95%CI)	P for	Studies	Ref.	RR (95%CI)	P for
	(n)			heterogeneity	(n)			heterogeneity
Histological subtypes								
EAC	9	[11-13,15,20,22,24,25,32]	1.42 (1.02-1.98)	0.19	8	[11-13,15,20,24,25,32]	1.38 (1.07-1.78)	0.3
ESCC	9	[11,12,16,17,19,21,23,27,29]	1.55 (1.10-2.17)		7	[11,12,16,19,21,27,33]	1.08 (0.80-1.44)	
Study location								
Asia	6	[14,17,18,19,23,26]	1.33 (1.09-1.62)	0.67	5	[19,33,34,36,37]	1.09 (0.61-1.95)	0.65
Europe	6	[11,13,20,21,27,30]	1.33 (0.86-2.07)		7	[11,13,20,21,27,30,35]	1.49 (0.99-2.23)	
United States	7	[12,15,22,24,25,31,32]	1.32 (1.03-1.70)		5	[12,15,21,25,32]	1.30 (1.08-1.57)	
South America	3	[16,28,29]	2.20 (0.48-10.04)		1	[16]	0.76 (0.51-1.13)	
Sex								
Male	3	[11,23,26]	1.26 (0.66-2.41)	0.88	2	[11,33]	1.24 (0.58-2.65)	0.14
Female	2	[11,23]	1.31 (0.78-2.21)		1	[11]	0.61 (0.33-1.13)	
Both	19	[12-22,24,25,27-32]	1.42 (1.17-1.71)		16	[12,13,15,16,19-21,24,25,27,30,32,34-37]	1.43 (1.15-1.77)	
Study quality <sup>1</sup>								
≥ 7	8	[11-14,16,18,20,22]	1.60 (1.20-2.13)	0.23	6	[11-13,16,20,25]	1.20 (0.88-1.62)	0.42
< 7	14	[15,17,19,21,23-32]	1.25 (1.02-1.54)		12	[15,19,21,24,27,30,32-37]	1.43 (1.11-1.86)	

<sup>1</sup>Study quality was assessed using the Newcastle-Ottawa Scale (range, 1-9 stars); RR: Relative risk; ESCC: Esophageal squamous cell carcinoma; EAC: Esophageal adenocarcinoma.

**A**



**B**

**Figure 2** The combined relative risks and 95%CI of esophageal cancer risk for the highest vs lowest categories of red meat (A) and processed meat (B). M: Male; F: Female; C: Combined males and females; ESCC: Esophageal squamous cell carcinoma; EAC: Esophageal adenocarcinoma.

In a meta-analysis of the 15 case-control studies, we found that the highest categories of processed meat intake were associated with a 36% increase in esophageal cancer risk when compared with the lowest categories (95%CI: 1.07-1.74; Figure 2B); however, we found a non-significant, positive association when we examined only the cohort studies (RR: 1.25; 95%CI: 0.83-1.86). When we examined whether an individual study was the source of heterogeneity among either the cohort or case-control studies, there were heterogeneities between the case-control studies ( $P < 0.01$ ,  $I^2 = 57.1\%$ ) and the cohort studies ( $P = 0.01$ ,  $I^2 = 63.4\%$ ). When the results from the cohort and case-control studies were combined, the overall combined RR comparing the highest and the lowest category of processed meat was 1.32 (95%CI: 1.08-1.62;  $P$  for heterogeneity:  $P < 0.01$ ,  $I^2 = 58.4\%$ ). The heterogeneity observed between the prospective studies of processed meat intake and esophageal cancer risk was no longer significant ( $P = 0.12$ ) after excluding a study by González *et al.*<sup>[13]</sup>. However, excluding any one case-control study from the analysis did not influence the heterogeneity findings observed among case-control studies.

No publication bias was found for either the cohort or case-control studies ( $P = 0.65$  for the cohort studies and  $P = 0.80$  for the case-control studies). In a dose-response meta-analysis of 3 cohort studies, we found

that each 100 g/d increase in processed meat intake was positively, but not significantly, associated with esophageal cancer risk (RR: 1.37; 95%CI: 0.88-2.13). There was no evidence of heterogeneity ( $P = 0.17$ ,  $I^2 = 33.5\%$ ).

When stratifying the analyses by histological subtypes, study location, sex, and study quality, we found no significant differences in the associations, although the magnitude of the associations differed slightly in these subgroups (Table 2). The associations also did not vary by adjusted confounding factors including alcohol, smoking, BMI, and fruit and vegetable intakes (data not shown).

## DISCUSSION

To our knowledge, this is the first systematic meta-analysis of cohort and case-control studies to summarize the evidence regarding the association between red or processed meat intake and the risk of esophageal cancer. High red meat consumption was associated with a 38% higher risk of esophageal cancer compared to low consumption in a meta-analysis of both case-control and cohort studies. A 26% higher risk of esophageal cancer was observed among those who had high red meat intake compared to those with low intake in a meta-analysis of 4 cohort studies. With regard to processed meat, we found a higher risk of esophageal cancer with high processed

meat intake compared to low intake in a meta-analysis of case-control studies, but the combined estimate of cohort studies did not reach statistical significance. Prospective cohort studies are less prone to selection or recall bias compared to case-control studies, which is critical in research of diet and cancer etiology. Therefore, a significant association in only the case-control studies and not in the meta-analysis of the 3 cohort studies could not provide adequate supportive evidence of an increased risk associated with processed meat consumption. However, the results for more prospective cohort studies need to be reported to obtain a clearer conclusion.

There are possible underlying mechanisms linking the consumption of red and processed meats and the incidence of cancer. HCAs and PAHs are chemical compounds with mutagenic potential that are formed when meat is boiled, fried, or grilled at high temperatures<sup>[3]</sup>. Animal studies have suggested that these two mutagenic compounds may induce changes in DNA, possibly promoting carcinogenesis<sup>[3,38]</sup>. Another class of meat-related mutagen is NOCs, the majority of which are potent carcinogens<sup>[39]</sup> formed either endogenously or exogenously. Processed meat is typically preserved by adding nitrate or nitrite, which increases the formation of NOCs<sup>[3]</sup>. Heme iron, largely derived from red meat sources, has been suggested to promote the endogenous formation of NOCs<sup>[40]</sup>. There is only limited epidemiological evidence, however, to suggest that the dietary intake of nitrite or nitrosamine is positively associated with the risk of esophageal cancer<sup>[5]</sup>. The esophagus is frequently exposed to these dietary mutagenic and/or carcinogenic compounds as stomach and colon, permitting food to pass from the esophagus into the stomach. While the specific mechanism by which meat causes esophageal cancer has not been fully elucidated, one likely reason may involve the potential for increase the susceptibility to carcinogenesis by repeated exposure of esophagus to the mutagenic and/or carcinogenic compounds, given their effects on carcinogenesis in animal models<sup>[3,38,39]</sup>.

The results from the subgroup and meta-regression analysis could not completely explain the potential sources of between-study heterogeneity because we did not observe statistically significant differences by histological subtype, study location, sex, or study quality. For red meat intake, it appeared that a single study did not substantially influence the overall combined RR, whereas, the observed heterogeneity among the prospective studies of processed meat intake and esophageal cancer risk disappeared when the study by González *et al.*<sup>[13]</sup> was excluded. However, the observed heterogeneity among the case-control studies of processed meat intake and esophageal cancer risk was not materially altered in sensitivity analyses excluding one study at a time.

Our meta-analysis had some limitations. Although the majority of the studies adjusted for known potential confounding factors, there may be a possibility that unidentified or residual confounding factors remained that were not adjusted for in the multivariate analysis or by covariates inadequately measured. Most studies, however,

adjusted for alcohol and smoking, both of which are established risk factors for esophageal cancer. Additionally, we found an increased risk of esophageal cancer with high red meat intake in a meta-analysis of well-scored studies, which were relatively recent and adjusted for various potential confounding factors. The random measurement error of meat consumption that occurred during dietary assessment or the systematic error resulting from recall or selection bias in the case-control studies may have influenced our findings; however, we found a statistically significant association between red meat intake and esophageal cancer risk in a meta-analysis of prospective studies, which supports the hypothesis that red meat intake increases the risk of esophageal cancer.

Our meta-analysis also included several strengths. Our meta-analysis updated the recent large prospective and case-control studies with a larger number of cases that were not included in previous reviews. In particular, the inclusion of new data from large cohort studies, which were unavailable when earlier conclusions of these associations were made by the WCRF/AICR expert panel<sup>[4]</sup> or by a review study<sup>[5]</sup>, enabled us to provide more unbiased evidence compared to the review that included only case-control studies. The findings from this meta-analysis were not subject to publication bias, indicating that the probability of publishing a study did not rely on the strength and direction of the associations.

## CONCLUSION

The findings from our meta-analysis of either prospective cohort or case-control studies suggest that a high consumption of red meat may increase the risk of esophageal cancer. Although we found an increased risk in a meta-analysis of the case-control studies for processed meat intake in relation to esophageal cancer risk, the prospective cohort studies did not strongly support this evidence. There is a need for further large scale prospective studies to determine whether processed meat intake increases the risk of esophageal cancer. Moreover, further studies evaluating the effect of red or processed meat intake on individual histological subtypes of esophageal cancer are warranted.

## ACKNOWLEDGMENTS

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## Current trends in the development and application of molecular technologies for cancer epigenetics

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### Abstract

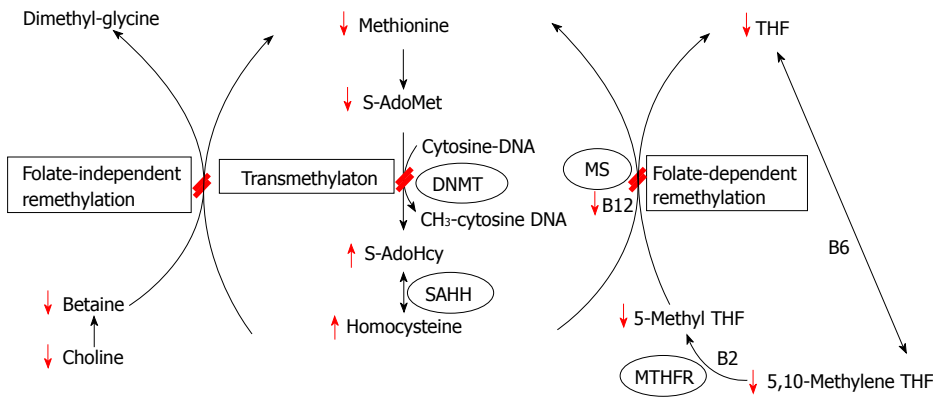
Current progress in epigenetic research supports the view that diet and dietary components are important in cancer etiology by enhancing or inhibiting carcinogenesis. Since diet and dietary factors may significantly contribute to the causation and progression of many cancers, it is important to find the molecular mechanisms of action of such dietary factors for cancer prevention and treatment. Recently, the role of epigenetic mechanisms in the cancer development and progression has attracted more attention as additional evidence along with traditional DNA sequence based mechanisms such as mutations and structural re-arrangements. Such an increasing interest in cancer epigenetics has also accelerated the development and application of molecular assays and tools for DNA methylation detection and histone modification enrichment analysis. In this paper, key assays and methods for epigenetic research are reviewed and discussed in terms of their utility and usability. In addition, more advanced methods for genome-wide analysis are introduced as part of upcoming research trends and directions.

### INTRODUCTION

Diet and dietary factors play an important role in many biological processes and are also involved in the regulation of pathological progressions including cancers. Several epidemiological and preclinical studies suggested that increased intake of bioactive dietary components may modulate cancer risk. Many studies provide compelling evidence that part of the anti-cancer properties contributed to several bioactive dietary components may relate to modulation of epigenetic process including DNA methylation and histone protein modifications. Here, we provide a brief overview of dietary modulation of DNA methylation and histone modifications and its potential role in cancer prevention. Also, we will discuss several new epigenetic methods to help understand the effect of dietary factors on epigenetic modifications.

### EPIGENETICS AND CANCER

The growing interest in the role of epigenetics in cancer came from the demonstration that epigenetic modifications are involved in tumor development and progression. Epigenetics can be defined as phenomena that alter the expression of the information in the genome at the transcriptional, translational, or posttranslational



**Figure 1** Effect of methyl-deficiency on biological methylation pathway. S-AdoMet: S-adenosylmethionine; S-AdoHcy: S-adenosylhomocysteine; DNMT: DNA methyltransferase; SAHH: S-adenosylhomocysteine hydrolase; MS: Methionine synthase; THF: Tetrahydrofolate; MTHFR: Methylenetetrahydrofolate reductase.

level without change in DNA sequence<sup>[1,2]</sup>. Epigenetic information is maintained to preserve cellular identity in normal cells, while cancer cells are characterized by profound alteration of epigenetic regulation<sup>[3-7]</sup>. The overall disruption of epigenetic phenomena is a common feature of all human tumors and includes alteration of DNA methylation and histone modification patterns<sup>[8]</sup>. DNA methylation patterns of neoplastic cells have been recognized as being substantially altered compared with normal cells<sup>[3,4]</sup>. Two types of changes in the DNA methylation pattern can occur in cancer: global DNA hypomethylation and hypermethylation of CpG islands which are associated with gene silencing<sup>[3,4,7]</sup>. DNA in eukaryotic cells is intimately associated with a family of small, basic histone proteins forming a highly ordered and condensed DNA-protein complex termed chromatin. Because of this chromatin structure, changes in DNA methylation in cancer cells are not isolated events; they occur in the context of more complex epigenetic deregulation<sup>[9]</sup>. Chromatin is the physiological template of the genetic information and is composed of DNA, histones, and other chromosomal proteins. The fundamental repeating unit of chromatin is the nucleosome octamer, which consists of 147 base pairs of DNA wrapped around 2 copies each of histones H2A, H2B, H3 and H4<sup>[10]</sup>. The amino-terminal tails of histones are subject to posttranslational modifications, including acetylation, methylation, phosphorylation, ubiquitination, SUMOylation, and ADP-ribosylation<sup>[11]</sup>, and multiple histone modifications may occur on a given histone tail<sup>[12]</sup>. Histone modifications patterns distinguish the structure of chromatin status, in particular, acetylation of histone H3 and H4 is associated with active gene expression with open chromatin structure. Histone acetylation is regulated by several enzymes such as histone acetyltransferase and histone deacetylases activity. Aberrations in post-translational modifications of histones have been shown to occur in cancer cells. Although alterations in global histone modification patterns in cancer cells have remained unknown, recent studies on global histone modifications at specific amino acids have been suggested as predictive clinical outcomes for various cancers<sup>[13-15]</sup>. Additionally, a number

of studies have been focused only on changes of a particular histone modification at individual gene promoters in cancer cells.

## DNA METHYLATION

### *Methyl-deficient diet induced hepatocarcinogenesis*

In last 4 decades, researchers have developed various tools for exploring DNA methylation, and started to apply those new technologies to the field of nutrition science. The methyl-deficient model of endogenous hepatocarcinogenesis is one of which in DNA methylation has been extensively studied. This animal model is unique in that dietary omission rather than chemical carcinogens addition can lead to tumor formation<sup>[16]</sup>. Specifically, deficiency of the major dietary sources of methyl groups - methionine, choline, folic acid and vitamin B12 - leads to the development of liver cancers in rats and certain mouse strains<sup>[17-19]</sup>. From early 1990, these animal models have shown that the methyl-deficiency is associated with several defects, including genome-wide DNA hypomethylation and gene-specific hypermethylation<sup>[20-22]</sup>. Importantly, the aberrant epigenetic alterations imposed by this diet have been hypothesized to be the primary mechanism responsible for malignant transformation of rat liver cells<sup>[15,20,22,23]</sup>. Figure 1 displays a simplified version of biological methylation pathway from one-carbon metabolism, emphasizing that various dietary methyl sources (methionine, choline, various coenzymatic forms of folate and vitamin B2, B6 and B12) play an important roles in DNA methylation. Methyl source deficiency has marked effects on the flow of one-carbon units through this web of reactions as the effect of methyl source deficiency are highlighted in red. The major effect observed in methyl deficiency models is a rapid decrease in hepatic S-adenosylmethionine (SAdoMet) levels and genomic DNA hypomethylation. In other recent studies examining the early stages of hepatocarcinogenesis induced by methyl deficiency in rats, substantial alterations in other aspects of the epigenetic machinery have been observed, including aberrant expression of DNA methyltransferases and methyl-CpG

**Table 1** DNA methylation analyses used in methyl-deficient model of hepatocarcinogenesis in rodents

Dietary component	Model	Observations	Methylation assay	Ref.
Amino acid-defined diet lacking choline, methionine, folic acid and vitamin B12	Rat	Depletion of SAdoMet and DNA hypomethylation	Liver DNA methyltransferase activity assay with labeled SAdoMet	[20]
Amino acid-defined diet lacking choline, methionine, folic acid and vitamin B12	Rat	Hypomethylation of CCGG site of c-myc, c-fos and c-Ha-ras	Enzyme digestion by <i>Hpa</i> II / <i>Msp</i> I	[21]
Diet low in methionine lacking in choline and folic acid	Rat	Hypermethylation of p16 <sup>INK4A</sup>	MS-PCR	[94]
Diet low in methionine lacking in choline and folic acid	Rat	Decrease in the total percent of methylated CCGG sites in DNA	<i>Hpa</i> II / <i>Msp</i> I -based cytosine extension assay	[22]
Diet low in methionine lacking in choline and folic acid	Rat	Depletion of S-AdoMet, decrease in S-AdoMet/S-AdoHcy and global DNA hypomethylation	<i>Hpa</i> II / <i>Msp</i> I -based cytosine extension assay	[23]
Diet low in methionine lacking in choline and folic acid	Rat	Hypomethylation of ID element and LINE-1 in preneoplastic livers and liver tumors; Decrease in histone H4-Lys20 trimethylation and increase in histone H3-Lys9 trimethylation; Decrease in histone H4-Lys20 trimethylation at the LINE-1 regulatory region	ID methylation by methylation-sensitive MsrBC-PCR array; LINE-1 methylation by COBRA-assay; global histone methylation by Western blotting; LINE-1-associated histone methylation by ChIP	[15]
Diet deficient in methionine lacking in choline and folic acid	Rat	Changes in the DNA methylation machinery	Indirect methods by DNA methyltransferases and Methyl CpG binding proteins	[24]
Amino acid-defined diet lacking choline	Rat	Hypermethylation of upstream of E-cadherin and Cx26	Bisulfite sequencing	[95]
Diet deficient in methionine lacking in choline and folic acid	Rat	global loss of DNA methylation; hypermethylation of CpG islands	Global DNA methylation by cytosine extension assay and [ <sup>3</sup> H-methyl] incorporation; CpG island methylation by [ <sup>32</sup> P]dGTP incorporation	[30]
Diet deficient in methionine lacking in choline and folic acid	Mouse	Global DNA hypomethylation; substantial loss of repetitive sequences (LINE-1, SINES, IAP elements) cytosine methylation. Increase in histone H3-Lys9 trimethylation and decrease in histone H4-Lys20 trimethylation	Global DNA methylation by cytosine extension assay; methylation-sensitive MsrBC-qPCR assay; global histone modifications by Western blot	[96]
Diet deficient in methionine lacking in choline and folic acid	Mouse	Detection of CpG island methylation profiles	MeDIP	[97]

S-AdoMet: S-adenosylmethionine; ID: Identifier; LINE-1: Long interspersed nucleotide elements; SINES: Short interspersed nuclear elements; IAP: Intracisternal A-particle; MS-PCR: Methylation-specific polymerase chain reaction; ChIP: Chromatin immunoprecipitation; MeDIP: Methylated DNA immunoprecipitation.

binding proteins<sup>[24]</sup>, defects in histone methyltransferase protein expression and histone posttranslational modifications<sup>[15]</sup>. In Table 1, various DNA methylation assays were summarized in methyl-deficient model of hepatocarcinogenesis in rodents.

### Genomic DNA methylation assays

One of widely used methods for global DNA methylation assay is a radioassay that utilizes the enzyme *Sss* I DNA methyltransferase to catalyze the *de novo* methylation of the CpG sites with radiolabeled [<sup>3</sup>H]-SAdoMet, a universal methyl donor *in vitro*<sup>[20,25-28]</sup>. And another method was developed thanks to the discovery of methylation-sensitive restriction endonucleases. In 1999, Pogribny *et al.*<sup>[29]</sup> developed a new method based on methylation-sensitive endonucleases followed by single nucleotide extension with radiolabeled [<sup>3</sup>H]-dCTP. This cytosine extension assay was used in various studies of methyl-deficient model of hepatocarcinogenesis for genomic DNA methylation<sup>[22,23,29,30]</sup>. These enzyme based methods have wide variations in precision as a result of inconsistency in the activity of methyl-sensitive endonucleases and the instability of methyltransferase activity<sup>[31]</sup>. In 2002, Friso *et al.*<sup>[32]</sup> developed a method for quantitative determination of 5-methyl-2'deoxyctidine using liquid chromatogra-

phy/electrospray ionization/mass spectrometry (LC/ESI/MS). This method allows accurate measurement of the absolute amount of 5-methyl-2'deoxyctidine relative to the total amount of cytosine residues, furthermore, it requires relatively lower amount of DNA and has a shorter run time for each sample than other high-performance liquid chromatography-based methods<sup>[33-35]</sup>. DNA methylation assay by LC/ESI/MS has been widely used for quantitative DNA methylation in animal studies and population-based studies in the light of its greater reproducibility and precision in large number of samples<sup>[32,36-40]</sup>.

### Gene-specific DNA methylation measurements

DNA methylation has long been recognized as an important factor on the silencing of genes, therefore it has become important to know the methylation status of individual CpG site. The first generation of DNA methylation detection assay is Southern blot or polymerase chain reaction (PCR) amplification that follows the enzyme digestion with methylation-sensitive restriction endonucleases<sup>[41-46]</sup>. Currently, the most commonly used methods for gene-specific DNA methylation can be categorized into three major methods.

### Bisulfite DNA sequencing and methylation-specific

**PCR:** Treatment of DNA with bisulfite converts cytosine residue to uracil, but leaves 5-methylcytosine residue unaffected. Bisulfite sequencing involves chemical conversion of cytosine to uracil, followed by PCR, and DNA sequencing<sup>[47]</sup>. While providing single-base resolution, the high cost and labor-intensive steps limit the use of this method for high-throughput analyses<sup>[48]</sup>. Methylation-specific-PCR also employs bisulfite conversion, but avoids the need to sequence the area of interest. Instead, methyl-specific and unmethyl-specific primer sets are designed, to distinguish methylated from unmethylated DNA in bisulfite-converted DNA<sup>[49]</sup>. This method is powerful to explore CpG islands with high methylation density, as increased numbers of CpG in the primer increase the specificity of the assay. However, these two methods using bisulfite conversion are not currently suitable for whole-genome analysis on multiple samples but commonly used for data validation from array-based methods.

**Methods that focus specific single-CpG:** These include Combined Bisulfite Restriction Analysis (COBRA)<sup>[50]</sup>, MethyLight<sup>[51]</sup>, and bisulfite pyrosequencing<sup>[52]</sup>. In COBRA, the combination of bisulfite conversion and PCR amplification is used, therefore it results in sequence conversion (unmethylated cytosine residue to thymidine and methylated cytosine to cytosine) which can lead to new methylation-dependent restriction enzyme sites. The following digestion of the PCR product with at least one CpG site in the recognition sequence only proceeds if the CpG site is protected from bisulfite conversion by methylation. For this reason, the signal ratio of restriction products indicating methylation to undigested PCR product representing unmethylated sequences can be used as a measure for the methylation level of this specific CpG. MethyLight is a bisulfite-dependent, fluorescence-based, quantitative real-time PCR method for DNA methylation. MethyLight relies on methylation-specific priming combined with methylation-specific fluorescent probing. This combination of methylation-specific detection principles results in a highly methylation-specific detection technology, with an accompanying ability to sensitively detect very low frequencies of hypermethylated alleles. Bisulfite pyrosequencing has been used to analyze bisulfite-converted DNA without using methylation-specific PCR. Following PCR amplification of the region of interest, pyrosequencing is used to determine the bisulfite-converted sequence of specific CpG sites in the region. The ratio of cytosine to thymidine at individual sites can be determined quantitatively based on the amount of cytosine and thymidine incorporation during the sequence extension. While the methods mentioned above are sensitive, specific, and relatively inexpensive, none of these methods is suitable for analysis of the whole genome, which includes about 28 million CpGs.

**Microarray-based methods:** These enable to interro-

gate larger numbers of CpG, there are three major types of microarray-based methylation analysis. Direct hybridization to CpG island arrays is the first high-throughput approach capable of detecting DNA methylation in genes across several CpG sites. Based on the bisulfite modification of DNA, this method utilizes methylation-specific oligonucleotides arrayed on glass slides for detection of all possible methylation in target DNA<sup>[53]</sup>. Methylated DNA immunoprecipitation (MeDIP) is also a large-scale, genome-wide purification method that is used to enrich for methylated DNA sequence using antibody raised against 5-methylcytosine<sup>[54]</sup>. DNA from MeDIP can be used for either array-based hybridization (MeDIP-chip) or high-throughput sequencing (MeDIP-seq). Although MeDIP helps generate comprehensive DNA methylation profiles, both applications have their typical limitation of array-based technology, restricted resolution. The HELP assay (*Hpa*II tiny fragment enrichment by ligation-mediated PCR) is comparative isoschizomer profiling of DNA methylation<sup>[55]</sup>. DNA is digested by *Hpa*II in parallel with *Msp*I (resistant to DNA methylation), and then the *Hpa*II and *Msp*I products are either amplified by ligation-mediated PCR and hybridized using separate fluorochromes to a customized array, or directly sequenced<sup>[56]</sup>. These high-throughput array-based approaches for DNA methylation are relatively inexpensive tool suitable for genome-wide analysis, therefore, help to target aberrant methylation patterns in various cancer models. Furthermore, methylation profiling achieved from high throughput methods will offer differentially methylated regions to understand the effect of dietary factors on epigenetic modifications in cancer, subsequently, provide insight in prevention strategies to reduce the burden of cancer.

## HISTONE MODIFICATION

### *Histone deacetylase inhibition by butyrate*

In addition to the effects on DNA methylation, dietary components can affect posttranslational modifications of histones. The dietary agent best studied in histone modifications is the short chain fatty acid butyrate which is generated in the colon as a result of bacterial fermentation of dietary fiber. Higher intake of dietary fiber is associated with reduced risk of colorectal cancer<sup>[57,58]</sup>. The molecular mechanisms underlying this anti-cancer effect of dietary fiber are poorly understood, however, the strongest evidence is based on the anti-carcinogenic actions of butyrate. Butyrate can be found at millimolar concentrations in the lumen of the colon<sup>[59]</sup>, and has inhibitory effects on types I and II histone deacetylase enzymes. Butyrate-induced alterations in histone marks, especially acetylation at histone H3 and/or H4, have been associated with several processes, including cellular differentiation<sup>[60,61]</sup>, cell cycle arrest<sup>[62-64]</sup>, apoptosis<sup>[65-67]</sup>, and inhibition of invasion<sup>[68]</sup> in a number of cancer cell studies. Table 2 summarized some of evidence of the effects of butyrate on histone acetylation. Although butyrate has strong marks



**Table 2** A summary of selected evidence for effects of butyrate in histone modification and histone modification assays in cancer cell culture models

Dietary component	Cell culture model	Observations	Histone modification assay	Ref.
Sodium butyrate	SW620 human colon carcinoma cells	Increased global histone H4 acetylation	Western blot	[70]
Sodium butyrate	A375 human melanoma and S91 mouse melanoma	Increased global histone H4 acetylation	Western blot	[98]
Sodium butyrate	Colo-320 human colon cancer cells	Increased acetylation of histone H3 and H4 within CDKN1A promoter site	ChIP	[64]
Sodium butyrate	EBC-1 human lung epithelial cells	Increased histone H3 and H4 acetylation associated with promoter of cathelicidin	ChIP	[99]
Sodium butyrate	HepG2 human hepatocarcinoma	Increased global histone H3 and H4 acetylation; Genome-wide changes in acetylation of DNA-bound histones	Western blot; ChIP-chip (ChIP and microarray hybridization)	[100]

CDKN1A: Cyclin-dependent kinase inhibitor 1A; ChIP: Chromatin immunoprecipitation.

on histone acetylation, a small fraction of cellular genes is regulated in response to butyrate<sup>[69-71]</sup>. Therefore, it should be noted that site-specific approach by chromatin immunoprecipitation (ChIP) based experimental tools will provide a better understanding on the chemopreventive effects of butyrate, showing gene-specific histone acetylation and its associated gene expression.

### Global histone acetylation assays

The first estimates for the rate of acetylation turnover were measured by pulse, pulse-chase, and steady-state acetylation labeling in hepatoma tissue culture cells in 1975<sup>[72]</sup>. Boffa *et al.*<sup>[73]</sup> showed that sodium butyrate suppressed histone deacetylation *in vivo* and *in vitro* by measuring the kinetics of [<sup>3</sup>H] acetate release from histone proteins. Since specific antibodies to modified histones were developed, Western blot has been used to detect histone modifications. As shown in Table 2, butyrate-induced histone acetylation was confirmed by Western blot in many studies.

### Gene-specific histone acetylation measurements

**ChIP:** The antibodies to acetylated histone H3 and H4 have been used for ChIP to determine histone acetylation in specific regions of gene promoter and other regulatory regions. ChIP is a specialized immunoprecipitation used to detect the covalent interaction between the DNA sequence and DNA-binding proteins such as transcription factors or histone proteins. ChIP using histone antibodies is able to determine the specific location in the genome that various histone modifications are associated with, indicating the target of the histone modifiers<sup>[74]</sup>. For example, ChIP experiment unveiled that butyrate induced an increase in histone H3 and H4 acetylation within the CDKN1A promoter, which regulates the p21 protein, in Colo-320 human colon cancer cells<sup>[64]</sup>. Due to its ability to precisely detect the DNA binding of modified histones, transcription factors, and non-histone chromosomal proteins, ChIP has been widely used to generate and test numerous hypotheses regarding transcriptional and epigenetic regulations. However, it remains to be still challenging to conduct ChIP on

an “epigenome” level, since ensuring an antibody of high specificity is often laborious and time-consuming. Another important concern with ChIP scalability is the maximum range of target regions that can be investigated by a single assay. For instance, a typical experiment of ChIP coupled with qPCR is designed to measure the enrichment levels of a DNA binding protein at a handful of sites (*e.g.*, gene promoters). However, in general, even a single epigenetic event in the cell pervasively occurs over a wide range of genomic regions, often involving thousands of genes and their associated regulatory elements. Thus, it becomes more important to have an ability to run the assay on a genome-wide scale for having a more balanced and unbiased perspective on the underlying mechanisms. Coupled with genomic profiling technologies such as tiling arrays or next generation sequencing (NGS), ChIP can be extended over the whole genome. In the following sections, we will introduce two major methods coupled with ChIP that enable epigenome-scale research of histone marks and transcription factors.

**ChIP-chip:** ChIP-chip is based on the combination of ChIP and a genomic tiling array technology (*i.e.*, chip), in which DNA sequences extracted after ChIP hybridize with probes that are designed to cover the whole genome or specific regions of interest such as promoter<sup>[75,76]</sup>. Due to bias in microarray hybridization, a control experiment using chromatin input or DNA from non-specific immunoprecipitation (IP) (*e.g.*, IP against immunoglobulin G) is often recommended. Most algorithms for ChIP-chip are designed to compute the normalized ratio between the hybridizations of ChIP and control after removing random and/or systemic noise. Then, they call binding sites as those significantly enriched in ChIP over control<sup>[77-79]</sup>. Since its emergence in the early and mid 2000s, ChIP-chip has been widely adopted in many transcriptional and epigenetic regulation studies, assisting scientists to more understand the role of each histone mark in physiological and pathological processes<sup>[80-83]</sup>. However, the utility of ChIP-chip is heavily restricted by a tiling array probe design, which determines the resolution of

the measurement (*i.e.*, intervals between adjacent probes) and the regions that can be explored (*e.g.*, omission of repetitive sequence areas). These weaknesses of ChIP-chip have accelerated the major platform shift to NGS.

**ChIP-Seq:** In ChIP-Seq, the extracted DNA sequences are directly sequenced using a NGS technology instead of being hybridized onto tiling arrays. NGS refers to sequencing technologies that newly emerged since the mid 2000s as an alternative to the traditional automated Sanger sequencing. NGS is characterized as massive parallel sequencing of template DNA or RNA (cDNA) molecules by a relatively short length ranging over 50-400 bp<sup>[84]</sup>. One advantage of ChIP-Seq over ChIP-chip is that ChIP-Seq does not require any predefined array design, which allows a more unbiased assay at a much higher resolution (100-1000 bp in ChIP-chip *vs* 10-100 bp in ChIP-Seq). Since NGS generally produces a notoriously large amount of data than array-based methods, more powerful bioinformatics support is essential for data processing and analysis<sup>[85,86]</sup>. Bioinformatics analysis for ChIP-Seq in epigenetic research includes the pre-processing for sequence data such as quality control and read mapping, the identification of candidate sites enriched by the target histone mark, and further down-stream analysis for revealing biological implications of the observations from the precedent steps<sup>[85,86]</sup>.

In cancer studies, the down-stream analysis is focused on finding the most associated genes or regulatory elements (*e.g.*, promoters or enhancers) with the histone mark of interest and investigating how these genes and regulatory elements can be understood in the context of biological pathways. Since Barski *et al.*<sup>[87]</sup> and Wang *et al.*<sup>[88]</sup> studies on 19 histone methylations and 18 histone acetylations using the human CD4+ T cell, many studies have been done to understand the biological implications of histone marks in normal conditions<sup>[80-82]</sup>. However, due to the plasticity of epigenome and heterogeneity of cancer, cancer epigenetics of examining histone modifications on a genome scale still remains in its beginning stage. For this reason, most of currently on-going efforts in cancer epigenetics still largely target DNA methylation (*e.g.*, The Cancer Genome Atlas, <http://cancergenome.nih.gov/>)<sup>[89]</sup>. Therefore, it will be a long-term goal to accumulate the knowledge on cancer epigenetics from histone modifications and use it for cancer studies, which will require a great amount of public and private investments. Another interesting research direction is an attempt to comprehend how genetic variations lead to epigenetic changes in cancer. In 2011, several studies have been published about the possibility of multiple chromatin remodelers and histone enzymes as potential oncogenes or tumor suppressor genes<sup>[90-93]</sup>. These studies suggest that the disruption of chromatin remodelers and histone enzymes due to driving somatic mutations in their coding regions may cause aberrant epigenetic changes, which eventually lead to cancer development or evolution in at least several cancer indications. Such approach is particularly interesting because it may be able to provide a genu-

ine perspective on the target histone mark by observing somatic mutations in several key chromatin remodelers and histone enzymes.

## CONCLUSION

In conclusion, a number of aberrant epigenetic modifications have been found in cancer cells, and diet and dietary factors play an important role to prevent cancer as well as to stimulate carcinogenesis. The use of epigenetic technology offers significant advantages to study the epigenetic mechanisms of cancer development and progression. Also, the newly developed technologies for epigenetic study expand the scope of nutrition study in the field of cancer research by helping monitor and pin down specific epigenetic pathways in diet-related cancers.

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## Use of thiopurines in inflammatory bowel disease

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### DOSING AND MONITORING OF THIOPURINES

Thiopurines used for inflammatory bowel disease (IBD) treatment are azathioprine (AZA), 6-mercaptopurine (6-MP) and occasionally also 6-thioguanine (6-TG). The most commonly used thiopurine is AZA. The hepatic enzyme glutathione-S-transferase rapidly cleaves the pro-drug AZA to 6-MP<sup>[1]</sup>, which is then metabolized in both liver and gut by several enzymes: (1) thiopurine-s-methyltransferase (TPMT) catalysing 6-MP to 6-methyl-MP (6-MMP); (2) xanthine oxidase catalyzing 6-MP to thiourea; and (3) hypoxanthine-guanine-phosphoribosyltransferase converting 6-MP to 6-thioguanine nucleotides (6-TGN). 6-TG is the final effector-metabolite<sup>[2]</sup> which slowly accumulates in cells, and this metabolite is probably responsible for the delayed onset of action after 10-12 wk<sup>[3]</sup>.

Dose recommendations for AZA and 6-MP vary slightly between Western guidelines, with a daily dose of 2-3 mg/kg AZA and 1-1.5 mg/kg 6-MP recommended by the AGA<sup>[4]</sup>, and a daily dose of 1.5-2.5 mg/kg AZA and 0.75-1.5 mg/kg 6-MP recommended by the European Crohn's and Colitis Organisation (ECCO)<sup>[5]</sup>. However, these recommendations do not necessarily hold true for other ethnicities. Several Japanese studies showed that Japanese IBD patients might reach sufficient 6-TGN values with substantially lower AZA and 6-MP dosages in adults<sup>[6,7]</sup>, children and adolescents<sup>[8]</sup>. If 6-TG is exceptionally used, it should be started at a much lower dosage of approximately 20 mg per day and should not exceed

### Abstract

The use of thiopurines as immunosuppression for the treatment of refractory or chronic active inflammatory bowel disease is established for both Crohn's disease and ulcerative colitis. Nevertheless, many questions remain concerning the optimal treatment regimens of azathioprine, 6-mercaptopurine and thioguanine. We will briefly summarize dose recommendations, indications for thiopurine therapy and side effects which are relevant in clinical practice. We discuss some currently debated topics, including the combination of azathioprine and allopurinol, switching of thiopurine therapy in case of side effects, the use of azathioprine in pregnancy, the infection risk using thiopurines and the evidence when to stop thiopurines. Excellent reviews have been published on the thiopurine metabolic pathway which will not be discussed here in detail.

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**Key words:** Thiopurines; Inflammatory bowel disease;

25 mg daily<sup>[9]</sup>. However, it has to be strengthened that there are limited indications for use of 6-TG in IBD. 6-TG can be used in IBD patients who need a thiopurine for maintenance therapy but who are intolerant (or resistant) to 5-aminosalicylic acid (5-ASA) [in ulcerative colitis (UC)], AZA, 6-MP and methotrexate [in Crohn's disease (CD)], and who do not have an option for surgery<sup>[9]</sup>. Biological therapy could be considered as an alternative to 6-TG in this setting. The ECCO guidelines<sup>[5]</sup> concluded in 2006 that thioguanine cannot currently be recommended for maintenance of CD due to a high frequency of liver abnormalities (mainly nodular regenerative hyperplasia). According to the current literature, this might, however, be explained by the use of too high dosages of 6-TG resulting in 6-TGN levels exceeding 1000 pmol/8 × 10<sup>8</sup> erythrocytes which is much more than the target levels of 250-500 pmol/8 × 10<sup>8</sup> erythrocytes usually recommended when using AZA or 6-MP<sup>[10-12]</sup>. Thus, if 6-TG is used as therapy for selected IBD patients, the 6-TGN levels should be controlled regularly combined with close monitoring of liver values<sup>[13]</sup>, hematology and especially liver biopsy after one, three and then every three years<sup>[9]</sup>. However, the histopathological diagnosis of nodular regenerative hyperplasia is difficult with a very poor inter-observer agreement, even among experts<sup>[14]</sup>. Alternatively, non-invasive monitoring such as magnetic resonance imaging may be considered<sup>[15]</sup>.

The dose recommendations as mentioned above are based on "Western" or "Caucasian" guidelines/evidence and are linked to the assumption that the TPMT pathway is "normal". A diminished TPMT activity due to mutations of the *TPMT* gene leads to toxic levels of 6-TGN, which is a risk factor for drug-induced myelosuppression<sup>[16]</sup>. TPMT mutations are not uncommon in Caucasian populations (mutation prevalence approximately 10%), but rarer in *e.g.*, Chinese patients (5%) or even very rare in Japanese patients (1%)<sup>[17]</sup>, which questions the usefulness of routine TPMT monitoring in these patients. The role of pharmacogenetics has recently been addressed in an excellent review by Chouchana *et al.*<sup>[18]</sup> and is shortly summarized below.

To achieve an optimal therapy, it seems crucial to find the correct concentration of intracellular 6-TGN (serum levels are of no value). Concentrations that are too high lead to myelosuppression, whereas concentrations that are too low result in a lack of efficacy in IBD<sup>[16,19]</sup>. TGN-levels can be measured irrespective of the time of intake of thiopurines. The commonly recommended 6-TGN concentration is 235-500 pmol/8 × 10<sup>8</sup> erythrocytes. In a meta-analysis<sup>[20]</sup>, patients with 6-TGN levels above the threshold value of 235 were more likely to be in remission than those below the threshold value (62% *vs* 36%, *P* < 0.001). The usefulness of therapeutic drug monitoring has recently been supported in a Dutch study<sup>[21]</sup>. However, the minimum effective dose is unknown. No dose-response study has ever been carried out, but at least one study showed that an increase in AZA dosage (2.02-2.72 mg/kg daily) can induce response in some patients<sup>[22]</sup>. Since there is only a very weak correlation between thio-

purine dosage and 6-TGN levels<sup>[20]</sup> and some ethnic variability<sup>[6,7]</sup>, the authors believe that 6-TGN levels should be regularly measured. Measuring the 6-TGN levels alleviates uncertainties when AZA therapy is combined with 5-ASA in UC<sup>[23]</sup>. In a recent and well-performed prospective study, the addition of 2 g of 5-ASA increased the total amount of 6-TGN by 50%, and methylmercaptopurine ribonucleotide levels were reduced after adding 4 g of 5-ASA<sup>[24]</sup>. Further studies are needed to show whether 6-TGN measurements might be useful to improve thiopurine therapy.

## TPMT MONITORING

Beside the above mentioned measurement of thiopurine metabolites, TPMT genotyping (analysis of single nucleotide polymorphisms which influence the TPMT activity) and phenotyping (measuring the TPMT activity) can be applied before therapy with AZA or 6-MP is initiated. A recent study concluded that genotyping should be favoured for pre-treatment TPMT function since it is the more reliable test<sup>[25]</sup>. This evaluation may help to identify slow metabolizers which are at risk of toxicity<sup>[4]</sup>. Nevertheless, a recently meta-analysis stated that there is not yet enough evidence to advocate for routine TPMT testing<sup>[26]</sup>. The United States Food and Drug Administration (FDA), but not the ECCO, recommends TPMT monitoring before the initiation of thiopurine therapy. In 2011, the first guideline for a thiopurine starting dose according to TPMT phenotype/genotype was developed<sup>[27]</sup>. A reduction to 30%-70% of the full dose is recommended in patients with an intermediate activity (heterozygote). In patients with low/absent activity (homozygous mutant or compound heterozygote), an alternative drug should be considered or AZA starting dose must be reduced by a 10-fold, administered thrice weekly, and under close monitoring. For patients with intermediate and low/absent TPMT activity, a therapeutic drug monitoring is recommended four weeks after treatment initiation. Patients with a very high activity might benefit from an increase in AZA dosage up to 3.0 mg/kg per day<sup>[18]</sup>. Nevertheless, it is important to note that TPMT testing does not predict the long-term risk of myelosuppression or idiosyncratic adverse events such as fever, arthralgias, hepatitis or pancreatitis<sup>[28]</sup>. Regular hematologic monitoring remains necessary<sup>[26]</sup>, initially at least every second week until the patient has been on a stable dose for a month; in the later course at least every third month<sup>[29,30]</sup>, including a complete blood count including a differential count, especially to check the lymphocyte level but also platelets as well as amylase, and liver enzyme levels<sup>[4,30,31]</sup>.

## INDICATIONS FOR AZATHIOPRINE OR 6-MERCAPTOPURINE

### *No evidence for induction of remission in active IBD*

There is not enough evidence to recommend thiopurines



for inducing remission in active IBD<sup>[32]</sup> based on five randomized controlled trials (RCTs) in active CD<sup>[33-37]</sup> and on two RCTs in active UC<sup>[38,39]</sup>.

### Prevention of relapse in quiescent IBD

In quiescent CD, AZA and 6-MP are effective at preventing a relapse based on two RCTs comparing AZA with placebo<sup>[33,37]</sup>, and three RCTs comparing continued AZA *vs* withdrawal in patients who had been successfully maintained on AZA<sup>[40-42]</sup>. These studies showed a significantly higher relapse rate in the placebo groups as compared with the active treatment<sup>[32]</sup>. Additionally, a RCT evaluating glucocorticoid-dependent CD patients suggested that AZA was better than placebo at reducing the need for glucocorticoids<sup>[43]</sup>.

In quiescent UC, AZA and 6-MP are effective at preventing a relapse based on three RCTs comparing AZA with placebo<sup>[38,39,44]</sup>. Overall, 60% of patients can be kept in remission if AZA is continued for 9-12 mo<sup>[32]</sup>. Similarly, another RCT which evaluated AZA withdrawal in 79 patients, who had been maintained on this drug for a minimum of 6 mo, showed a significant reduction on the relapse rate on AZA *vs* placebo after one year (36% *vs* 59%)<sup>[45]</sup>.

### Post-operative treatment of CD with thiopurines

Two RCTs in postoperative CD suggest that AZA or 6-MP is superior to placebo at preventing recurrence after surgery<sup>[46,47]</sup>. Furthermore, thiopurines administered postoperatively significantly reduced the clinical recurrence rates in population-based cohorts<sup>[48,49]</sup>. Thus, thiopurines might be a useful strategy, although anti-tumour necrosis factors (anti-TNFs) might be more efficient in preventing CD relapses postoperatively<sup>[50,51]</sup>, especially among high risk patients (smokers, perforating disease or  $\geq$  second operation)<sup>[50]</sup>.

### Treatment of fistulising CD with thiopurines

A meta-analysis in fistulizing CD demonstrates a significantly higher response rate to AZA and 6-MP as compared to placebo (54% *vs* 21%)<sup>[52]</sup>. The analyzed studies included patients with perianal, enterocutaneous, enteroenteric and rectovaginal fistulas. However, only in a minority of patients, AZA and 6-MP will lead to a complete fistula closure, but symptoms such as inflammation, discharge and discomfort are often substantially reduced. Since response of fistulas to these drugs will need several months, immunosuppression will be reasonable only as second-line treatment if fistulas don't necessitate immediate surgery. Antibiotics are commonly used as first-line treatment and need to be started in parallel<sup>[53]</sup>.

An overview of indications for thiopurines in IBD is given in Table 1.

## ADVERSE EVENTS

AZA and 6-MP have similar side effects leading to dis-

continuation of therapy in 39% of patients in a large Dutch cohort<sup>[54]</sup>, however, rates of intolerance are usually far lower. Most adverse events occur within the first 3 mo<sup>[55]</sup>. More than 50% of AZA intolerant patients tolerate 6-MP long-term<sup>[56]</sup>. Common side effects can be divided into dose-dependent and idiosyncratic side effects.

The major dose-dependent side effect of thiopurines is drug-induced myelosuppression which is observed in 2%-5% of Caucasian patients<sup>[57,58]</sup>. It can occur at any time with 25% of cases appearing beyond the first year<sup>[55]</sup>. Asian (or at least Japanese patients<sup>[6,7]</sup>) have a higher risk of myelotoxicity. It has been speculated that viral infections might contribute to myelosuppression, however, clear evidence is missing. Determining TPMT activity before starting thiopurines might avoid early myelosuppression, but regular haematological monitoring remains necessary in every patient.

Infectious complications are mostly dose-dependent, but sometimes idiosyncratic side effects. Infectious complications during thiopurine therapy can occur even in the absence of a dose-dependent leukopenia<sup>[4,59,60]</sup>, especially when using a combination of thiopurines with corticosteroids which may induce a dose-dependent lymphocyte depletion. We recommend avoiding a lymphopenia of  $< 600/\mu\text{L}$  based on data from a rheumatological study<sup>[31]</sup>. Doing this, severe immunodeficiency is likely to be avoided<sup>[31]</sup>. Hepatotoxicity can manifest as early drug-induced hepatitis, nodular regenerative hyperplasia after years of therapy, sinusoidal dilatation or fibrosis<sup>[61]</sup>. It is important to note that IBD *per se* is a risk factor for nodular regenerative hyperplasia<sup>[62]</sup>. Hepatotoxicity induced by thiopurines seems more often dose dependent than idiosyncratic. In many patients, elevated transaminases respond to dose reduction. In a prospective monocentric cohort, 21 of 123 patients showed a transient or constant elevation of alanine aminotransferase levels<sup>[63]</sup>. If liver enzymes are repeatedly elevated, thiopurines need to be discontinued.

The most frequent idiosyncratic side effects are nausea, vomiting, and malaise in up to 15% of all patients<sup>[64]</sup>. Some advocate to slowly increase the dosage when thiopurine therapy is started, or to take it before night-time. However, the best way to start thiopurine therapy still has to be determined<sup>[4]</sup>. Other common side effects are headache, fatigue, anorexia, weight loss, stomatitis, alopecia, arthralgia, muscular weakness and rash, which may occur in more than 10% of patients. If these side effects are reported, it should be determined whether they disappear after dose reduction<sup>[4,59,60,65,66]</sup>. In case of arthralgias/myalgias upon AZA, a switch to 6-MP can be explored<sup>[55]</sup>.

Pancreatitis is an important idiosyncratic side effect and occurs in up to 4% of the patients<sup>[4,60]</sup>, especially during the first weeks of treatment<sup>[67]</sup>. A minor and asymptomatic increase in serum amylase ("pancreatic hyperenzymemia") is frequently observed, but only poorly understood and not discussed in current guidelines. Some authors prefer to reduce dosing, or stop treatment

**Table 1** Indications for thiopurines in inflammatory bowel disease

	Indication	No indication
Crohn's disease	Maintaining remission in moderate (to severe) CD (any site of disease especially for extensive disease)	Induction of remission (as a sole therapy in active disease)
	Maintaining remission in CD with early relapse (< 3 mo after the last flare) or frequent flares (more than two per year)	
	Fistulizing CD (in combination with antibiotics, if no early start of anti-TNF or surgery necessary)	
	Postoperative prevention of CD recurrence (unless high-risk situation such as repeated surgery or current smoker)	
Ulcerative colitis (treated with 5-ASA at optimal dose unless intolerant)	In combination with anti-TNFs in case of severe CD (rapid step-up or top-down)	Induction of remission (as a sole therapy in active disease)
	Maintaining remission in steroid-dependent UC	
	Maintaining remission in UC with early relapse requiring steroids	
	Maintaining remission in UC with frequent flares requiring steroids	
	Maintaining remission in UC after induction of remission by ciclosporin, tacrolimus, or <i>i.v.</i> steroids	
	Acute or chronic refractory pouchitis	

CD: Crohn's disease; UC: Ulcerative colitis; TNF: Tumour necrosis factor; 5-ASA: 5-aminosalicylic acid.

in case of lack of biochemical response<sup>[68]</sup>. Thiopurines must be discontinued if amylase increase is associated with typical pain symptoms (*i.e.*, toxic pancreatitis). After AZA-induced pancreatitis, a switch to 6-MP is not recommended since these patients are less likely to tolerate 6-MP<sup>[64]</sup>. However, evidence against the use of 6-MP in AZA-induced pancreatitis is weak. As earlier outlined, 6-TG is a debated alternative to AZA and 6-MP in case of intolerance<sup>[9]</sup>, which is, however, not recommended by some authors<sup>[69,70]</sup>.

## WHEN AND HOW TO CHANGE THIOPURINE THERAPY IN CASE OF SIDE EFFECTS?

In the case of idiosyncratic side effects, it might be reasonable to change from AZA to 6-MP, as discussed above. Furthermore, in so-called preferential 6-MMP metabolizers which achieve only low 6-TGN levels due to high 6-MMP levels with associated hepatotoxicity, adding low-dose allopurinol as a XO inhibitor to dose-decreased AZA (25%-33% of intended dose<sup>[71]</sup>, might switch the AZA metabolism to 6-TGN instead of 6MMP. Close monitoring of 6-TGN metabolites and hematology is in such cases necessary (weekly during the first month, then every other week for the next month)<sup>[69]</sup>. This strategy often allows to reach therapeutic levels of 6-TGN and clinical remission, but may also to reduce or even alleviate nausea as one of the major early side effects in more than 80% of patients<sup>[72]</sup>. Interestingly it seems possible to achieve higher 6-TGN and lower 6-MMP levels in preferential 6-MMP metabolizers by simply splitting the daily thiopurine dose<sup>[73]</sup> or by switching to 6-MP.

## MALIGNANT COMPLICATIONS

Treatment with AZA/6-MP is associated with a potential risk of developing lymphoma<sup>[74]</sup>, including hepatosplenic T-cell lymphoma (HSTCL)<sup>[75]</sup>. Although the relative risk of lymphoma is increased four to five-fold<sup>[74,76]</sup>, the absolute risk still remains rather small, and currently available data show that the benefits of thiopurines used in IBD greatly outweigh its risks<sup>[77]</sup>. The same holds true for

non-melanoma skin cancer<sup>[78-80]</sup>, which in a large cohort of 108.518 IBD patients collected from 1997 to 2009 was shown to occur significantly more often in IBD patients on thiopurines especially among those with CD than controls<sup>[79]</sup>, correlating with the length of receiving thiopurines. Of 32 cases of nonmelanoma skin cancer in a large cohort<sup>[80]</sup>, only 5 cancers occurred in immunomodulator-naïve patients, but 9 and 18 cancers occurred in patients who had previously taken or were currently on thiopurine therapy. Based on these studies, a dermatologic exam should be considered before and regularly during immunomodulator therapy; especially in elderly patients. It should in this context be mentioned that skin protection is rather crucial.

## WHEN SHOULD THIOPURINE MONOTHERAPY BE STOPPED?

When to stop a successful immunosuppression is one of the most difficult decisions in IBD therapy. Five studies focussing on this question were recently reviewed by Clarke and Regueiro<sup>[81]</sup>. According to a randomized, controlled study from 2005, even CD patients who were in remission on AZA for at least 3.5 year profited from prolonged AZA therapy (relapse risk 8% *vs* 21% on placebo)<sup>[40]</sup>. Similar results have been gained from other studies<sup>[82]</sup>. Five years after stopping AZA, 3/4 of patients suffer from relapse. Nevertheless, a treatment stop seems justified since almost all patients re-treated with thiopurines were able to regain remission (23 of 24 patients; many with a combined short course of glucocorticoids)<sup>[83]</sup>. Independent predictors for a flare are a C-reactive protein-level of > 20 mg/L, a haemoglobin-level < 12 mg/dL, and an absolute neutrophil count of > 4 × 10<sup>9</sup>/L at baseline. Since more than half of the patients with risk factors experienced a clinical flare-up within 24 mo (compared to only 15% of patients without negative predictors), a continuous course of thiopurine therapy is recommended for these patients.

In UC, several studies have shown that a prolonged AZA therapy helps to reduce the risk of relapse. Six months is certainly the minimum length of immunosuppression (with at least 3 mo disease-free interval off glu-

corticosteroids)<sup>[84,85]</sup>, but other studies showed that 18 mo is significantly better than 6 mo<sup>[81]</sup>.

In clinical practice, decision on the length of immunosuppressive therapy means weighing benefits of immunosuppression on IBD and risk of malignancies and other complications. The risk factors should be analysed before immunosuppression is stopped. Best candidates for stopping immunosuppression will probably be IBD patients in deep, prolonged remission with a short duration of time between diagnosis and immunomodulator treatment<sup>[81]</sup>. Nonetheless, prospective RCTs are needed for the development of evidence-based tapering schemes regarding AZA and 6-MP treatment, both in CD as well as in UC.

## IMMUNOMODULATORS AND BIOLOGIC THERAPY

Many experts in the field currently advocate a “top down” approach with early combination therapy for moderate to severe CD based on evidence from three infliximab and azathioprine studies in CD<sup>[86-88]</sup>, including the famous SONIC trial<sup>[86]</sup>. Nevertheless caution is advised as the number needed to treat for the combination therapy is 8, meaning that only one out of 8 patients will definitely benefit from the combination. For the combination of AZA and adalimumab, there are only retrospective and hardly convincing data<sup>[89]</sup>. Future trials need to determine whether it is crucial to start with anti-TNFs and thiopurines at the same time, or whether a sequential addition of thiopurines to anti-TNFs will have the same effect in the patients experiencing a benefit from this combination.

Furthermore, there are not enough available data on how long the concomitant use of immunomodulators should be maintained in patients receiving infliximab. There are no studies which documented a sustained efficacy of concomitant use of immunomodulators beyond 6-12 mo<sup>[90,91]</sup>. If therapy should be reduced, then AZA and not infliximab may be stopped. In the recently published prospective STORI (Stop Infliximab in Patients With Crohn's Disease) study on infliximab stop in patients in remission under combined immunosuppression, nearly half of all patients suffered from relapse within 1 year when infliximab was stopped despite continuing azathioprine<sup>[92]</sup>. In contrary, the risk of relapse after stopping AZA seems lower than after stopping infliximab. Thus, in a single referral center observational study on CD patients who stopped AZA after being in remission under combined AZA and infliximab for at least 6 mo, 15% of patients relapsed after 1 year<sup>[93]</sup>.

## THIOPURINES IN PREGNANCY AND LACTATION

Even though thiopurines belong to FDA pregnancy category D, the risk seems minimal. Two studies<sup>[94,95]</sup> did not find any evidence for an increased risk of pregnancy-re-

lated complications. Probably, the only major side effect of thiopurines is the risk of preterm birth<sup>[95-97]</sup>, which, however, might be simply a disease effect. Furthermore, thiopurine levels in breast milk of mothers treated with AZA seem harmless<sup>[98]</sup> and without any long-term effects in these babies. Thus, it can be concluded that thiopurines can be administered safely to women with IBD, prior to and at the time at conception, as well as during pregnancy and lactation. Indeed, in a recent survey among gastroenterologists showed that more than 90% would recommend continuous thiopurine treatment<sup>[99]</sup>.

## CONCLUSION

Indications for use of thiopurines in IBD and ways to monitor therapy have been well established. In clinical practice, the possibility of 6-TGN measurement to monitor therapy seems underused. It is likely that broader use of TGN monitoring, and a combination of thiopurines with low dose allopurinol in case of inefficiency or side effects allows to (achieve or) maintain clinical remission in more patients. For the thiopurine/allopurinol combination, but also for 6-TG therapy, prospective trials to document the benefit (and risk) are urgently needed. Furthermore, more trials are needed to elucidate whether an early “top down” approach for combination of thiopurines with anti-TNF will bring a long-term benefit for patients as compared to a step-up approach in case of a non-response to thiopurines. Criteria when a successful thiopurine therapy for maintenance of remission can be stopped also need to be elucidated in future prospective trials. Risk communication with patients, but also referring physicians, about benefit, side effects and the above mentioned uncertainties remain challenging. Benefits are the quality of life gained by medically maintained remission; the avoidance of surgery (in CD and UC) and avoidance of colorectal cancer through efficient anti-inflammatory therapy. Risks are, however, (opportunistic) infections, lymphomas such as HSTCL and side-effects such as pancreatitis.

Accordingly, thiopurine therapy remains a hot topic in IBD.

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## Tailoring the area of hepatic resection using inflow and outflow modulation

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Such important technical achievements should be a fundamental part of the surgical armamentarium of the modern liver surgeon.

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**Key words:** Hepatic resection; Intraoperative ultrasound; Liver inflow; Liver outflow; Resection guidance

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### Abstract

The performance of hepatic surgery without a parenchyma-sparing strategy carries significant risks for patient survival because of the not negligible occurrence of postoperative liver failure. The key factor of modern hepatic surgery is the use of the intraoperative ultrasound (IOUS), not only to stage the disease, but more importantly to guide resection with the specific aim to maximize the sparing of the functional parenchyma. Whether in patients with hepatocellular carcinoma and underlying liver cirrhosis, or in patients with colorectal liver metastasis, IOUS allows the performance of the so-called "radical but conservative surgery", which is the pivotal factor to offer a chance of cure to an increasing proportion of patients, who until few years ago were considered only for palliative care. Using some new IOUS-guided surgical maneuvers, which are based on the liver inflow and outflow modulations, more precise anatomically subsegmental- and segmental-oriented resections can be effectively performed. The present work describes the rationale and the surgical technique for a precise tailoring of the area of hepatic resection using the most recent attainments in IOUS.

### INTRODUCTION

The performance of hepatic surgery without a parenchyma-sparing strategy carries significant risks for patient survival because of the not negligible occurrence of postoperative liver failure, which is definitely related to the amount of the sacrificed parenchyma<sup>[1,2]</sup>. Indeed, major or extended hepatic resections are independent negative prognostic factors with regard to short- and long-term outcomes<sup>[2-7]</sup>. The key factor of modern hepatic surgery is the use of the intraoperative ultrasound (IOUS) not only to stage the disease, but more importantly to guide resection, with the specific aim of maximizing parenchyma-sparing, removing only the tumoral tissue<sup>[8]</sup>. Whether in patients with hepatocellular carcinoma (HCC) or in patients with colorectal liver metastasis (CLM), IOUS allows the performance of the so-called "conservative but radical surgery"<sup>[9]</sup>, which is the pivotal factor to offer a chance of cure to an increasing proportion of patients, who until few years ago were considered only for palliative care. Indeed, in cases of HCC with cirrhosis the underlying liver function is generally marginal, and the prognosis of the patient might be more related to the residual liver function rather than to the pres-



ence of HCC. In such patients, the hepatectomy should always be tailored on the basis of both tumoral features and functional liver reserve. Similarly, in cases of CLM the rationale of the surgical approach described here is based on the need to minimize the rate of major or extended resections with the aim of reducing operative risk, and at the same time preserving the liver parenchyma, which could be the site for future hepatic recurrence potentially re-treated with curative intent.

## OPERATIVE TECHNIQUE

The J-shaped laparotomy is the preferred incision for liver surgery, and the access into the right thoracic cavity following the 9<sup>th</sup> intercostal space is carried out to control the hepatocaval confluence. In particular, the thoracoabdominal approach is selected in obese patients, in patients with a deep chest, and during complex reoperations. Thus, the liver is partially mobilized by dividing the round and the falciform ligaments. Sometimes the coronary and triangular ligaments are also divided early to obtain enough space for IOUS. This should in fact be performed before complete mobilization of the liver to avoid any artifact made by the surgical maneuvers.

## INTRAOPERATIVE ULTRASOUND

IOUS is the procedure of choice to stage disease in patients with liver tumors. It should be fully performed by the surgeon in charge for the operation rather than by the assistants, radiologists or technicians. This is because the information gathered during the exploration requires interpretation to have most impact on the surgical strategy. Thus, IOUS is mainly performed to plan the surgical strategy rather than to locate the lesions. Generally, high frequency probes (7.5-10 MHz) are recommended for IOUS, since they allow for a higher spatial resolution than those working at lower frequencies (3.5-5 MHz). However, those latter probes are very useful for the initial exploration providing a better panoramic view. Lower frequency probes are also useful for allowing contrast-enhanced IOUS. Different shapes of probes are available for intraoperative use: the linear T-shaped, the inter-digital, and micro-convex probes. The best probe is the one that ensures the optimal compromise between the volume of the probe itself, which should be minimal, the scanning windows, which should be the largest, and the stability once in contact with the liver surface. In this sense, a new micro-linear probe with trapezoid scanning windows probably represents the best compromise among all the aforementioned requirements; this probe is furthermore designed to meet the requirements for those surgical maneuvers discussed here (Figure 1). Of note, the performance of IOUS may take time, and it requires experience to be effective and beneficial<sup>[10]</sup>.

## RESECTION GUIDANCE

Apart from staging, IOUS is essential to guide resection.

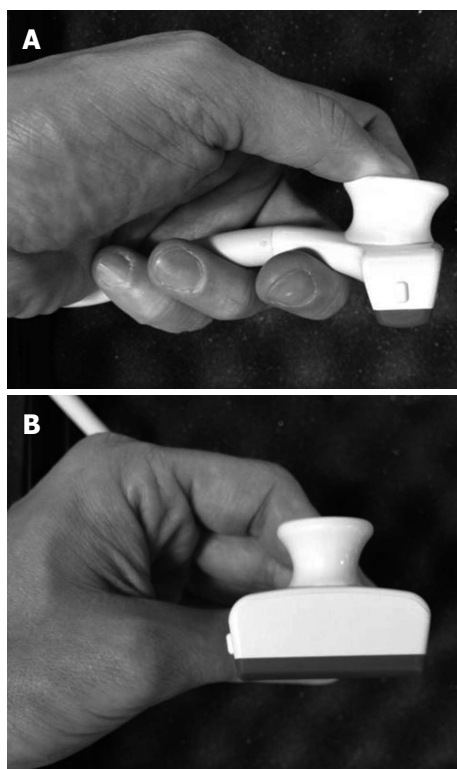
It is almost impossible to correctly define the hepatic segmental boundaries without IOUS, nor the boundaries of the tumor itself because of the existing wide variations in anatomy. The main advantage of IOUS-guided resection is modification of the traditional approach to liver tissue dissection, which involves dissection in vertical planes to avoid tumor exposure on the cut surface. With IOUS, the relationship between the dissection plane and the tumor edges can be followed in real time, and the direction of the dissection plane can be modified when needed. Versatile dissection planes around the tumors can avoid tumor exposure while sparing important vascular structures, thus sparing vital liver parenchyma. This approach has been recently redefined by the authors as the “radical but conservative approach”, and should be applied in liver surgery to maximize the results<sup>[9]</sup>. Also, in patients in whom major resections should be required, IOUS allows better design of the dissection plane, leading to conservative surgery even in patients with complex tumoral presentations<sup>[11]</sup>. Specific, and original IOUS techniques have already been developed to help the surgeon during the operation<sup>[12-15]</sup>. The following paragraphs will focus on two crucial techniques for defining the area of resection using IOUS findings.

## PLANNING OF THE SURGICAL STRATEGY

The information achieved from the preoperative imaging workup, which has an essential role in staging intra- and extra-hepatic disease, should be used to plan the surgical strategy. However, the surgical strategy should be intraoperatively defined only after IOUS exploration. The impact of IOUS on the operative decision-making, when compared with that of preoperative imaging techniques, is reported to be around 4%-7%<sup>[16,17]</sup>. These relatively low rates may be explained because of the different surgical policies applied by the different centers as well as the different tumor types considered. Indeed, IOUS, when used in a systematic and extensive way to map the tumor nodules, allows a 3-dimensional reconstruction of the relationships between the tumor and the main intrahepatic vascular structures [glissonian pedicles and hepatic veins (HVs)], which is pivotal in planning the individualized surgical strategy for each patient. Indeed, some experienced authors reported better results in terms of IOUS accuracy<sup>[18-21]</sup>. Some important tumor-vessel relationship rules have been developed by the authors, both for HCC and for CLM, with the aim of providing an intraoperative guide to individualize the surgical strategy and minimize parenchyma sacrifice.

### *Tumor in contact with a glissonian pedicle*

The glissonian pedicle may be spared when in contact with an encapsulated HCC or with a CLM once the integrity of the vessel wall is confirmed at IOUS, without any sign of distal bile duct dilation. For CLM, the contact should extend for less than one-third of the pedicle circumference. In the presence of bile duct dilation, tu-



**Figure 1** New probe for intraoperative ultrasound. This probe has a trapezoid scanning area, and an ergonomic shape, which help during intraoperative ultrasound-guided maneuvers. A: Lateral view; B: Front view.

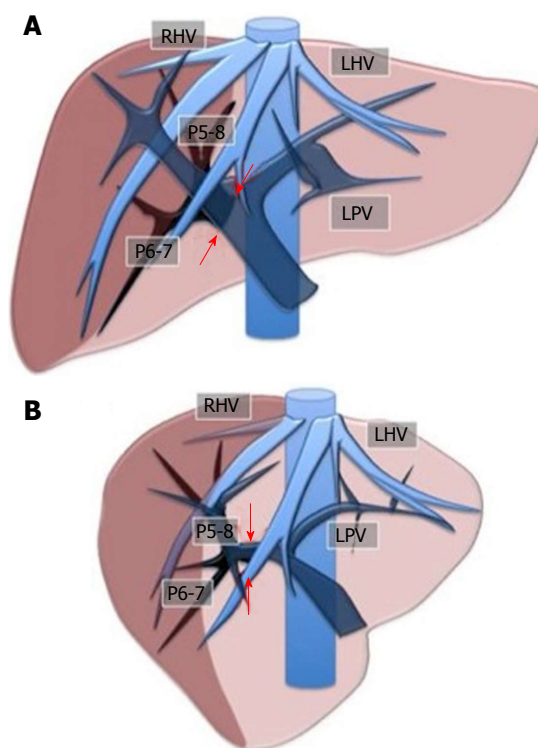
mor thrombus, or invasion of the vessel wall, the pedicle must be divided<sup>[9]</sup>.

### Tumor in contact with a HV

The HV may be spared when in contact with an encapsulated HCC or with CLM once the integrity of the vessel wall is confirmed at IOUS. For CLM, the contact should extent for less than two-thirds of the vessel circumference. Thus, in the presence of a tumor thrombus, invasion of the vessel wall, and wider contact the HV must be divided<sup>[15]</sup>. However, as described below, the extension of the hepatectomy to the portion of the liver theoretically drained by the resected HV is not systematically performed, but only when accessory HVs and/or communicating veins are missing or when inversion of the portal flow is demonstrated by IOUS<sup>[22]</sup>.

## INFLOW MODULATION

Initially used for tumors located in the left hemiliver<sup>[14]</sup>, the inflow modulation technique has more recently been successfully extended to any liver segment<sup>[23]</sup>, including segment 8, and even to sectional portions of the liver<sup>[24]</sup>. Once the feeding portal branch is identified at IOUS, it can be compressed using the IOUS probe by one side of the liver, and by the finger in the opposite side with the aim to induce a transient ischemia of the portion of the liver distal to the compression site. This portion can then be marked with the electrocautery, and when released, resection can be performed. This technique is

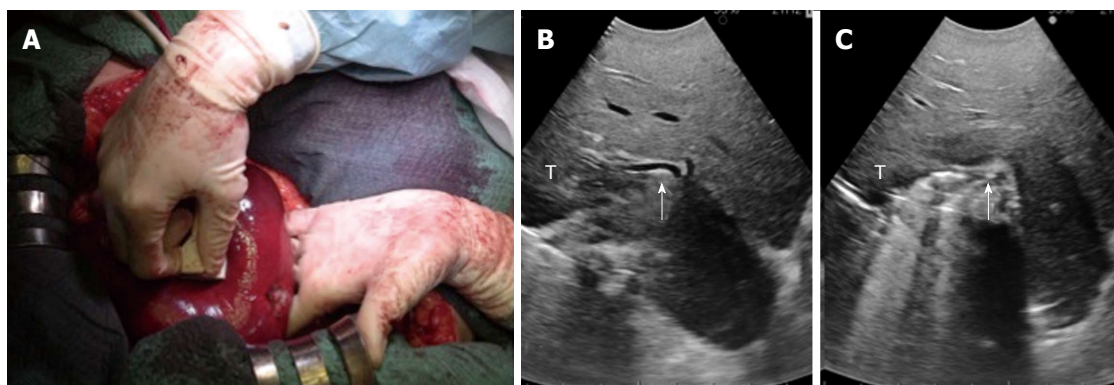


**Figure 2** Layout of the liver for the inflow modulation. Ischemic demarcation of the right posterior sector by intraoperative ultrasound-guided finger compression at its origin of the right portal bifurcation. A: Front view; B: Lateral view. RHV: Right hepatic vein; LHV: Left hepatic vein; LPV: Left portal vein; P5-8: Right anterior portal branches; P6-7: Right posterior portal branches. The arrows indicate the point for the compression.

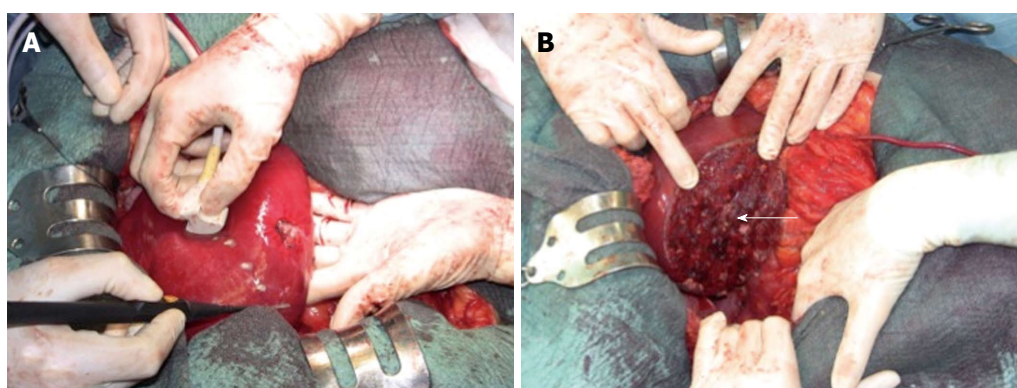
simple, fast, non-invasive, and reversible. Also, the possibility of modifying the site of the compression, and then the corresponding resection volume allows tailoring of the resection according to tumor features, and more importantly to the status of the background liver. This is of paramount importance in patients with HCC and cirrhosis, in which the functional liver reserve may be marginal. Such a technique allows for precise anatomical resection of a subsegment, segment or section of the liver. As is well-established, anatomical resection of HCC is recommended to offer a higher chance of cure<sup>[25-29]</sup>. For segments such as segment 1 and 4 superior, for which direct compression of the feeding portal branch may not be feasible, the compression of the adjacent segmental branches allows definition of their segmental margins. Indeed, our technique can be used in a counter-compression perspective similar to the counter-staining technique reported by Takayama *et al.*<sup>[30]</sup>. Figure 2 illustrates the layout of the liver with the compression technique applied to delineate the right posterior section, while Figures 3 and 4 show an actual case.

## OUTFLOW MODULATION

The area of resection may be tailored not only using US-guided finger compression of the portal branch as described above, but also using IOUS outflow modulation. Indeed, we have already showed how to minimize



**Figure 3** A case of intraoperative ultrasound-guided finger compression of segment 6. A: The portal pedicle for segment 6 is compressed by the probe in the right hand and by the finger in the left hand; B: Intraoperative ultrasound (IOUS) focused on the portal pedicle (arrow) for segment 6 before the compression; C: IOUS focused on the portal pedicle (arrow) for segment 6 during the compression. T: Tumor.



**Figure 4** Demarcation of the compressed area by electrocautery. A: The operative field before the resection; B: The operative field at the end of the resection. The arrow indicates the stump of the portal pedicle for segment 6.

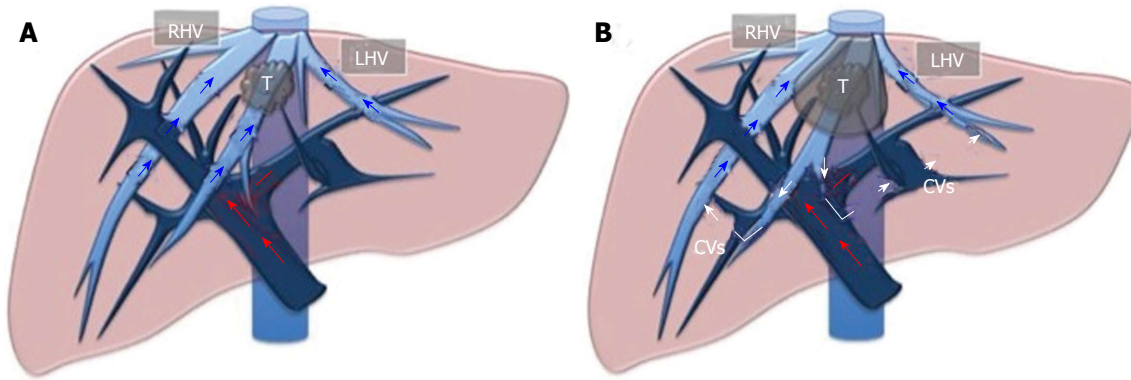
the sacrifice of liver parenchyma even in those patients with a tumor at the hepatocaval confluence, for which a standard major or extended hepatectomy should be indicated based on traditional criteria. In addition, we have introduced some new operations, such as minimesohepatectomy, and systematic extended right posterior sectionectomy<sup>[22-31]</sup>, which simultaneously limit the need for formal major resection, and improve the chances of resection for those patients with complex tumoral presentation. The definition of the resection area using the outflow control is based on the extensive use of IOUS flow analyses, with the aim of checking the outflow modifications once the HV that should be resected is clamped. For this purpose rather than the direct closure of the vein by a vessel loop, the US-guided fingertip compression at the caval confluence might be adequate<sup>[32]</sup>. Certainly, the HV may already be closed by the tumor. At that case, the search is focused on at least one of the following criteria: reversal of flow direction in the peripheral portion of the compressed HV, which suggests drainage through the collateral circulation in adjacent HVs or inferior vena cava (IVC); direct detection of collaterals between the compressed HV and adjacent HV or IVC; or persistence of hepatopetal flow in the portal branches corresponding to the area drained by the compressed HV. In particular, in the case

of hepatofugal flow direction in the portal branches, the resection should not be minimal but extended to the parenchyma fed by those portal branches. The presence of hepatofugal flow in the portal branches is a clear signal of insufficient drainage of the corresponding HV. Once at least one of the aforementioned criteria has been satisfied, full mobilization of the right and left hemiliver is performed, preserving most of the posterior short HVs to minimize the risk of congestion of the residual liver. Thus, the area of resection may be marked on the liver surface using electrocautery and IOUS to define the caudal, medial and lateral limits of the parenchyma to be removed, while the surgeon's left fingertip is visualized in the most cranial portion, and it is used to mark the dissection area. Parenchyma transection is then carried out with the surgeon's left hand behind the right hemiliver with the aim of guiding resection by the right hand in real time. Figure 5 illustrates the layout of the liver with the outflow modulation technique, while Figure 6 shows an actual case.

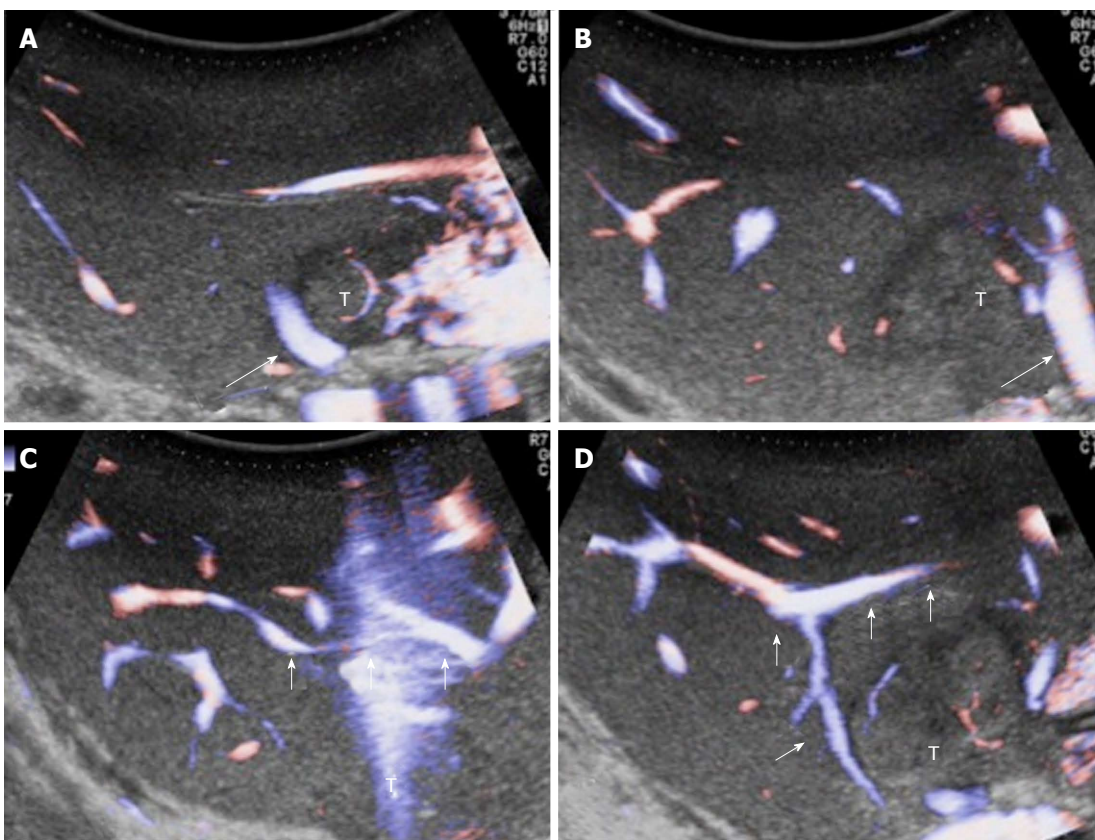
## PROBLEM OF THE SURGICAL MARGIN

One of the main criticisms of this surgical approach is the problem of the surgical margin. Both for HCC and CLM the detachment of the tumor from a spared vessel





**Figure 5** Layout of the liver for outflow modulation. A: A tumor in contact with the middle hepatic vein at the caval confluence; B: Once that vein is infiltrated and/or compressed, some collateral veins (CVs) shunting the flow from the middle hepatic vein territory to right hepatic vein (RHV) and/or left hepatic vein (LHV) territories can be detected. T: Tumor.



**Figure 6** Intraoperative ultrasound study of communicating veins. A: A tumour located between the middle hepatic vein (MHV) (arrow) and the left hepatic vein (LHV) at their confluence into the inferior vena cava; B: The arrow indicates the LHV; C, D: Evidence of communicating veins (arrows) between the LHV and the MHV. T: Tumor.

may mean zero millimeters surgical margin, which traditionally is classified as R1 resection by the pathologist. Indeed, exposure of the tumor on the dissection plane is sometimes required to spare intrahepatic major vascular structures, which is the mainstay of our surgical policy. However, the effect of surgical margin status on survival of patients with HCC and CLM has been studied, but controversy still remains among surgeons. There is still debate about the real impact of the extent of the surgical margin once tumoral tissue is removed from the cut

surface. For HCC, some authors reported that a margin smaller than 1 cm and even 2 cm plays a negative role in terms of long-term survival, while others authors found that a 0 mm margin is acceptable<sup>[33-37]</sup>. Also for CLM, there is no definitive agreement on the surgical margin<sup>[38,39]</sup>. It is well known that a positive margin is associated with increased risk of recurrence, but its width does not affect survival<sup>[40,41]</sup>. Moreover, it has been shown that patients with complex tumoral presentation treated with R1 resection may have the same long-term



survival of patients treated with R0 resection if aggressively treated with modern chemotherapy and repeated surgery<sup>[42]</sup>. Therefore, an anticipated minimal negative surgical margin should not be used as exclusion criterion for resection of HCC or CLM. The keystone is the performance of IOUS to guide the resection with the aim of achieving complete tumor clearance to minimize the risk of non-curative surgery.

In conclusions, IOUS is the best method for staging a liver tumor, and it is certainly the best method for the surgeon to understand in real-time the liver anatomy, and the relationships between tumors and intrahepatic vessels, thus allowing effective surgical operations. IOUS guidance allows for expanding indications offering the chance of cure to a greater proportion of patients, who would otherwise be excluded from the surgical program or submitted to more traditional but more risky operations. A precise tailoring of the area of hepatic resection using inflow and outflow modulation should be part of the surgical armamentarium of the modern liver surgeon.

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## ***Pseudomonas fluorescens*-like bacteria from the stomach: A microbiological and molecular study**

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### **Abstract**

**AIM:** To characterize oxidase- and urease-producing bacterial isolates, grown aerobically, that originated from antral biopsies of patients suffering from acid peptic diseases.

**METHODS:** A total of 258 antral biopsy specimens were subjected to isolation of bacteria followed by tests for oxidase and urease production, acid tolerance and aerobic growth. The selected isolates were further characterized by molecular techniques *viz.* amplifications for 16S rRNA using universal eubacterial and

*HSP60* gene specific primers. The amplicons were subjected to restriction analysis and partial sequencing. A phylogenetic tree was generated using unweighted pair group method with arithmetic mean (UPGMA) from evolutionary distance computed with bootstrap test of phylogeny. Assessment of acidity tolerance of bacteria isolated from antrum was performed using hydrochloric acid from  $10^{-7}$  mol/L to  $10^{-1}$  mol/L.

**RESULTS:** Of the 258 antral biopsy specimens collected from patients, 179 (69.4%) were positive for urease production by rapid urease test and 31% (80/258) yielded typical *Helicobacter pylori* (*H. pylori*) after 5-7 d of incubation under a microaerophilic environment. A total of 240 (93%) antral biopsies yielded homogeneous semi-translucent and small colonies after overnight incubation. The partial 16S rRNA sequences revealed that the isolates had 99% similarity with *Pseudomonas* species. A phylogenetic tree on the basis of 16S rRNA sequences denoted that JQ927226 and JQ927227 were likely to be related to *Pseudomonas fluorescens* (*P. fluorescens*). On the basis of *HSP60* sequences applied to the UPGMA phylogenetic tree, it was observed that isolated strains in an aerobic environment were likely to be *P. fluorescens*, and *HSP60* sequences had more discriminatory potential rather than 16S rRNA sequences. Interestingly, this bacterium was acid tolerant for hours at low pH. Further, a total of 250 (96.9%) genomic DNA samples of 258 biopsy specimens and DNA from 240 bacterial isolates were positive for the 613 bp amplicons by targeting *P. fluorescens*-specific conserved putative outer membrane protein gene sequences.

**CONCLUSION:** This study indicates that bacterial isolates from antral biopsies grown aerobically were *P. fluorescens*, and thus acid-tolerant bacteria other than *H. pylori* can also colonize the stomach and may be implicated in pathogenesis/protection.

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**Key words:** Antral biopsy; *Helicobacter pylori*; *Pseudomonas fluorescens*; HSP60; Nested polymerase chain reaction; Acid-tolerant bacteria

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## INTRODUCTION

*Helicobacter pylori* (*H. pylori*) is a gram-negative, microaerophilic bacterium found primarily in the stomach. It has been implicated in chronic gastritis, gastric ulcers, duodenal ulcers and stomach cancers that were previously believed to be of non-microbial origin<sup>[1-3]</sup>. There was a misconception that no bacteria could live in the stomach because of its highly acidic environment. For the first time, Steer and Colin-Jones<sup>[4]</sup> published their results regarding the presence of gram-negative, oxidase- and urease-producing bacteria, but they proposed that it was *Pseudomonas* (*P.*), a contaminant and not related to peptic ulcer. In an effort to grow *H. pylori* from an antral biopsy, we could see that a peculiar type of bacterial colony was growing consistently after overnight incubation while *H. pylori* was taking 3-5 d to grow. These colonies were also oxidase and urease producers, but growing in an aerobic environment. There are reports that show the presence of a variety of bacteria in the stomach by isolation, DNA profiling and polymerase chain reaction (PCR)-based analysis, and some of them are urease producers hindering the specificity of the urea breath test<sup>[5-7]</sup>. Therefore, we aimed to characterize this type of bacterial isolate and to analyze whether they are colonizers or contaminants.

## MATERIALS AND METHODS

### Collection of specimens

The study subjects were patients attending inpatient services of the Department of Gastroenterology, University Hospital of Banaras Hindu University, Varanasi, Uttar Pradesh, India. This hospital provides tertiary-level health services for the eastern part of Northern India. The culture isolation, phenotypic and molecular characterizations were carried out in the Department of Microbiology, Institute of Medical Sciences.

### Patients and samples

A total of 258 patients suffering from upper gastrointestinal (UGI) diseases like non-ulcer dyspepsia (NUD), peptic ulcer diseases (PUD) including gastric ulcer and duodenal ulcer, and gastric carcinoma were enrolled during a period of 3 years (2007-2010) and three antral biopsy pieces from each patient were collected. Before taking a biopsy, the endoscope was rinsed with detergent followed by water, and disinfected with 2% alkaline glutaraldehyde

for 30 min then rinsed with sterile water. In a similar way, biopsy forceps were washed and sterilized and one biopsy forceps was used for one patient exclusively. The biopsy specimens were collected by endoscopic forceps from each individual with full aseptic precautions after taking well-informed consent. The work was approved by the Ethics Committee of the Institute of Medical Sciences, Banaras Hindu University. Patients with mucosal breaks greater than 5 mm in size with apparent depth were diagnosed as having ulcer and those with ulcerato-infiltrative lesions with positive histology/brush cytology were considered as having stomach carcinoma. The patients not having ulcerative lesions but suffering from dyspeptic symptoms were diagnosed as NUD. Individuals with normal endoscopic findings without gastroduodenal symptoms but having other gut problems were treated as healthy controls. In the present study, those patients were excluded who had a history of previous gastric surgery, active UGI bleeding, chronic alcoholism, intake of antibiotics and proton pump inhibitors during the last 4 wk or those taking non-steroidal anti-inflammatory drugs. Further individuals less than 18 years of age, pregnant or lactating mothers or those having illnesses like cirrhosis, chronic renal failure or ischemic heart disease were also excluded.

### Microbiological processing

The three biopsy pieces were pooled and homogenized into phosphate saline buffer together in an all glass disposable homogenizer and were divided into three aliquots. The first aliquot of the tissue homogenate was transferred immediately into a rapid urease test (RUT) medium and the second was plated within 30 min of collection onto the media used for bacterial culture [Mueller Hinton agar without supplement and media containing brain heart infusion agar (Difco, Becton Dickinson, Sparks, MD, United States), supplemented with 7% sheep blood, 0.4% IsoVitalEX, and Skirrow selective supplement (vancomycin 10 µg/mL; polymixin B sulfate 2.5 IU/mL; trimethoprim lactate 5 µg/mL) (Difco, Becton Dickinson, Sparks, MD, United States)]. The non-enriched plate was incubated at 37 °C in an aerobic atmosphere while the other was incubated in the presence of 5% O<sub>2</sub>, 10% CO<sub>2</sub>, and 85% N<sub>2</sub> for 3-7 d. Several small colonies could be seen after overnight incubation in the first plate. The other plate was examined every alternate day after 3-7 d to see if colonies other than those observed after overnight incubation developed. Small translucent colonies developed after 5-7 d of incubation other than those originally after observed overnight incubation on non-enriched nonselective medium. These colonies were further subjected to morphological and biochemical tests viz. motility, oxidase, catalase, and urease. All the isolates were divided into two groups on the basis of the incubation period, i.e., those isolates obtained after overnight incubation designated as group A and the other group B possessing those strains isolated after 5-7 d of incubation. The third aliquot was subjected to genomic DNA extraction.



**Table 1** Primers used to study *Helicobacter pylori* and *Pseudomonas fluorescens* isolates

Target gene	Primer name	Primer sequence (5'-3')	PCR condition (number of cycles, size of product)
16S rRNA	16S F 16S R	TTGGAGAGTTTGATCCTGGCC ACGTCATCCCACCTTCTC	94 °C, 30 s; 59 °C, 30 s; 72 °C, 30 s (30, 1155 bp)
HSP60			
Primary	HSP1 HSP2	AAGGCATGCAATTGATAGAGGCT CTTTTTCTCTTTTCATTCCACTT	94 °C, 30 s; 56 °C, 30 s; 72 °C, 30 s (35, 594 bp)
Nested	HSPN1 HSPN2	TTGATAGAGGCTACCTCTCC TGCATAATCGCTTGTCTGTC	94 °C, 30 s; 56 °C, 30 s; 72 °C, 30 s (35, 501 bp)
Putative membrane-bound protein (PFMP)			
Primary	PFMPF PFMPR	TCTKRYCRMGAATCRARACWRYC GKTWYTGCKCRWWKCSYTSMMC	94 °C, 1 min; 50 °C, 1 min; 72 °C, 1 min (35, 704 bp)
Nested	PFMPNF PFMPNR	TGCGYWMWCCYWRWCCWTGA AKCABGGTSCWGMVRCRBC	94 °C, 1 min; 50 °C, 1 min; 72 °C, 1 min (35, 613 bp)
mupV			
Primary	mupVF mupVR	TGAGTTCGATGTGACCTGCCTG AACTCGCCAGATTGTCGTACAC	94 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min (35 cycles, 722 bp)
Nested	mupVnF mupVnR	CAGCATTATCTGCCACTGAC ATGATGTCTTGGCACACCTGATC	94 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min (35, 611 bp)

PCR: Polymerase chain reaction.

### RUT

For the RUT, biopsy specimens were inoculated into 1 mL of 10% urea in deionized water (pH 6.8), to which two drops of 1% phenol red solution was added, and incubated at 37 °C for 24 h. A positive result was recorded when the color changed from yellow to pink within 30 min. If there was mild color change within 30 min, the RUT tubes were incubated for a further 24 h.

### Subculture at different temperatures

The organisms which appeared after overnight incubation were tested for growth at 4 °C, 35 °C and 42 °C onto Muller Hinton agar; incubation was maintained for 14 d to distinguish *Pseudomonas* spp.<sup>[8,9]</sup>. Those organisms showing growth at 4 °C were sub cultured twice and incubated at the appropriate temperature for more than 10 d each time.

### Assessment of acidity tolerance of bacteria isolated from gastric niche

Five isolates were suspended into different molar concentrations of hydrochloric acid ( $10^{-1}$  to  $10^{-7}$  mol/L corresponding to pH 1.0 to 7.0) and a CFU was maintained as  $10^6$  CFU/mL. After the intervals of 0 min, 5 min, 10 min, 20 min, 30 min and 60 min, 100 µL acidic suspension ( $10^5$  CFU) were transferred into 3 mL BHI broth. After overnight incubation optical density was recorded with the help of a spectrophotometer at 600 nm wavelength and bacterial count was expressed in CFU/mL. This experiment was repeated twice.

### Preparation of genomic DNA for PCR assay

Extraction of genomic DNA from both types of bacterial isolates (A total of 320 strains including groups A + B) as well as from tissue homogenate was performed using a standard proteinase K and phenol-chloroform method<sup>[10]</sup>. To exclude the possibility of cross contamination

of DNA during DNA extraction, one set of double distilled was included in each batch of DNA extraction.

### Detection of *H. pylori* by nested PCR

Confirmation of *H. pylori* was done at a molecular level by nested PCR targeting the conserved *HSP60* gene and its restriction fragment length polymorphism. The reaction was performed in 25 µL final volume containing 10 ng of DNA, 1 U of Taq polymerase (Bangalore Genie, India), 200 mmol/L (each) deoxynucleotide triphosphate (MBI, Fermentas) and 1.5 mmol/L MgCl<sub>2</sub> in standard PCR buffer and 10 pmol of each primer as described by Singh *et al*<sup>[11]</sup>. Primer sequences and PCR conditions are displayed in Table 1. For the internal amplification, the PCR product from the primary cycle was diluted 1/50 and 1 µL was used as the template in the nested PCR. The conditions for the PCR amplification, first and nested reactions were the same. DNA from *H. pylori* reference J99 and a tube containing water in place of DNA were assayed in each PCR run as positive and negative controls, respectively.

After amplification, the PCR products (501 bp) were precipitated with 2.5 volumes of ethanol. The pellets were washed twice with 70% ethanol and dissolved in Tris-EDTA buffer (pH 8.0). A 10 µL precipitated amplified DNA sample was then digested with 10 U of restriction enzyme *Hind*III in appropriate buffered solution recommended by the manufacturer (Bangalore Genie, India) and incubated for 3 h at 37 °C. The digested DNA fragments were analyzed by electrophoresis on 2% agarose gels (Bangalore Genie, India) containing 0.5 µg of ethidium bromide per mL. The gel was run at 70 V with TBE (Tris Boric acid EDTA) buffer for 3 h and was examined by a transilluminator and photographed. The sizes of digested DNA fragments were estimated from distances of molecular weight standards and compared with *in silico* restriction digestion.

### Amplified rDNA restriction analysis

For each group of isolates, the 16S rRNA from 8 randomly selected isolates was amplified using forward primer-16SF and reverse primer-16SR<sup>[12]</sup>. PCR amplification was performed in a thermocycler (Biometra, Germany) according to standard procedures (Table 1). After amplification by universal eubacterial primers, the PCR products (1155 bp) were precipitated with 2.5 volumes of ethanol. The pellets were washed twice with 70% ethanol and dissolved in Tris-EDTA buffer (pH 8.0). A 10 µL precipitated amplified DNA sample was then digested with 10 U of restriction enzyme *Eco*R1, in appropriate buffered solution recommended by the manufacturer (Bangalore Genie) and incubated for 3 h at 37 °C. The visualization of the restriction fragment was done by the same method as described in the previous paragraph.

### Sequencing

The amplified PCR products were purified from salts and primers using QIA quick PCR purification kit (Qiagen, United States). A total of 8 purified amplicons generated targeting *HSP60* and 2 amplicon 16S rRNA genes were outsourced for partial sequencing to Bangalore Genie, India. Sequences were analyzed using BLASTN (<http://www.ncbi.nlm.nih.gov/BLAST>) to verify the identity of the sequences: whether *H. pylori* or some other microorganism.

### Sequence analyses

Reference sequences of *Pseudomonas* group and other enteric pathogens used for phylogenetic analyses were retrieved from Genbank. The partial 16S rRNA sequences and *HSP60* sequence for the strains were aligned with reference sequences using Clustal X version 1.81, with default parameters<sup>[13]</sup>. Phylogenetic and molecular evolutionary analyses were performed using *MEGA* version 4<sup>[14]</sup>. The phylogenetic tree was generated using the unweighted pair group method with arithmetic mean (UPGMA) from evolutionary distance computed with bootstrap test of phylogeny. The degree of statistical support for branches was determined with 500 bootstrap replicates.

### Primers designed for *Pseudomonas fluorescens* targeting putative membrane-bound protein

The putative membrane-bound protein coding gene present in all the strains of *P. fluorescens* is available in NCBI Genbank. Due to a similarity of sequences of about 55% in *P. fluorescens*, we therefore planned to design nested degenerate primers to amplify the partial sequence of putative membrane bound protein so that it was able to amplify all strains of *P. fluorescens*. Forward and reverse oligo-nucleotide degenerate primers derived from the region located between bases 92072 and 92775 of the *Pseudomonas fluorescens* (*Pseudomonas fluorescens* Pf0-1; GenBank Accession number CP000094.2 and GI: 253992019) were synthesized. Internal primers were derived from the region between bases 92114-92726 for nested PCR. DNA extracted from *P. putida* and *P. aeruginosa* was used to monitor sensitivity against *P.*

*putida* and *P. aeruginosa*. During the PCR assay in each batch, Mili Q water was used as a template to ensure that there was no contamination by water and PCR reagent<sup>[15]</sup>.

### *Pseudomonas fluorescens* NCIMB 10586 mupirocin biosynthetic gene

Forward and reverse oligonucleotides were derived from the conserved region located between bases 68101 and 68822 of the mupirocin biosynthetic gene cluster of *Pseudomonas fluorescens* (NCIMB 10586). An internal primer was derived from the region between bases 68796 and 68796 (GenBank Accession number AF318063.2; gene GI: 20150006). PCR amplification was similar to amplification of *HSP60* gene and conditions are described in Table 1.

### Randomly amplified polymorphic DNA PCR

Fingerprinting of 71 randomly selected strains from group A was performed based on randomly amplified polymorphic DNA (RAPD) PCR methods by using primers RAPD3 5'-TACAGCTCG-3' and RAPD5 5'-AGCACTGCCT-3' (this study). PCR was carried out in 25 µL volume using 10 ng of genomic DNA, 1 U of Taq polymerase (Bangalore Genie, India), and 15 pmol of each primer (Bangalore Genie), 200 mmol/L (each) deoxynucleotide triphosphate (Bangalore Genie, India) and 2 mmol/L MgCl<sub>2</sub> in standard PCR buffer. Amplification reactions were carried out in a thermal cycler (Biometra, Goettingen, Germany).

The gel images were analyzed under ultraviolet light in a gel documentation system (Alpha Innotech, United States). Cluster analysis of all the isolates was done on the basis of the fingerprint generated. Based on the presence or absence of different DNA fragments in the fingerprints of the *P. fluorescens* strains, a binary data matrix was created. Overall similarity between the pair of strains was calculated from the binary data matrix using the simple matching-dice coefficient. The resulting similarity matrix was used as the input data for cluster analysis by NTSYS pc2.0 programme of UPGMA<sup>[16]</sup>.

### Statistical analysis

The level of significance between the two proportions, *i.e.*, culture rates and molecular detection rates, was calculated by Fischer's Exact Probability test.

## RESULTS

### Bacteriological findings

Of the 258 antral biopsy specimens collected from patients, 69.4% (179/258) were found to be positive by RUT and 31% (80/258) by culture for typical *H. pylori*, after 5-7 d of incubation under a microaerophilic environment; these were gram-negative curved rods and were positive for oxidase, catalase and urease. However, 258 antral biopsies yielded 240 (93%) homogeneous semi-translucent and small colonies after overnight incubation. These isolates also grew aerobically but the colonies had

**Table 2** Comparative isolation rates and prevalence of *Helicobacter pylori* and *Pseudomonas fluorescens* against rapid urease test in antral biopsies by nested polymerase chain reaction targeting *HSP60* gene and membrane bound protein *n* (%)

Diseases	Antral biopsies	RUT, positivity	<i>H. pylori</i> <i>HSP60</i> , positivity	<i>P</i> value <sup>1</sup>	<i>P. fluorescens</i> putative outer membrane protein, positivity	<i>P</i> value <sup>2</sup>	Isolation of different types of bacteria, positivity		<i>P</i> value <sup>3</sup>
							Group A	Group B	
PUD	65	51 (78.5)	59 (90.8)	< 0.001	63 (96.9)	< 0.010	61 (93.8)	23 (35.4)	< 0.001
NUD	123	92 (74.8)	109 (88.6)	< 0.001	121 (98.4)	< 0.001	119 (96.7)	39 (31.7)	< 0.001
CA	49	23 (46.9)	24 (48.9)	< 0.050	46 (93.9)	< 0.001	43 (87.5)	12 (24.5)	< 0.001
Normal	21	13 (61.9)	19 (90.4)	< 0.001	20 (95.2)	0.001	17 (80.9)	6 (28.6)	< 0.001
Total	258	179 (69.4)	211 (81.8)	< 0.001	250 (96.9)	< 0.001	240 (93.0)	80 (31.0)	< 0.001

<sup>1</sup>*P* < 0.001 between *Helicobacter pylori* (*H. pylori*) and nested polymerase chain reaction (PCR); <sup>2</sup>*P* < 0.001 between *H. pylori* and *Pseudomonas fluorescens* (*P. fluorescens*); <sup>3</sup>*P* < 0.001 between colonies appearing after overnight incubation group (group A) and colonies appearing after 3-7 d of incubation group (group B). Group A: Non-ulcer dyspepsia (NUD) vs gastric carcinoma (CA), *P* = 0.02; NUD vs normal, *P* < 0.02; *H. pylori*: Peptic ulcer diseases (PUD) vs CA, *P* < 0.001; NUD vs CA, *P* < 0.001; CA vs Normal, *P* = 0.001; Overall: Culture of *P. fluorescens* vs nested PCR, *P* < 0.001. Normal: Patients whose endoscopic findings were normal; RUT: Rapid urease test; RUT: Rapid urease test.

an opaque, small character contrary to the translucent one which is typical for *H. pylori* (Table 2).

### Biochemical characterization

A total of 100 isolates randomly selected from group A were subjected to extensive phenotypic characterization. All of them were gram-negative, oxidase-, catalase- and urease-positive. All were non-fermenters, showed variable nitrate reduction and failed to utilize simple sugars (glucose, lactose, sucrose, mannitol and maltose). Citrate was utilized by all of them. All the tested strains were Methyl Red negative and Voges-Praskauer negative or equivocal. All the strains were oxidase- and catalase-positive. Indole test was negative but on mixing with the Kovac's reagent a typical greenish color developed. All these isolates were able to multiply at 4 °C. These findings intimated that those isolates which appeared after overnight incubation were *P. fluorescens*.

### Acid tolerance assay

The bacterial count of *P. fluorescens* was  $7.3 \times 10^8$ ,  $6.9 \times 10^8$ ,  $7 \times 10^8$ ,  $6.1 \times 10^8$ ,  $7.6 \times 10^8$ ,  $6.5 \times 10^8$  and  $9.8 \times 10^8$  CFU/mL for 0 min, 5 min, 10 min, 15 min, 20 min, 30 min and 60 min after acid exposure (pH 1.0). Average bacterial counts were  $9.3 \times 10^8$ ,  $9.8 \times 10^8$ ,  $1.0 \times 10^9$ ,  $9.5 \times 10^8$  and  $9.8 \times 10^8$  CFU/mL for acid tolerance of low pH 2, pH 3, pH 4, pH 5, pH 6 and pH 7, respectively, for different time intervals (0 min, 5 min, 10 min, 15 min, 20 min, 30 min and 60 min). Similarly, bacterial growth was approximately the same in control experiments where acidic solution was replaced by LB Broth. The exposure to acidic pH showed that *P. fluorescens* growth was not killed by exposure to lower pH 1.0 for an hour.

### Amplification and RLFP of HSP60 gene

Isolates from group B, which grew under microaerophilic environment, were subjected to amplification by primers specific for *HSP60* gene of *H. pylori*. All the 80 isolates from 258 patients were positive for the 501 bp of amplicon for the corresponding gene. However, 211 (81.8%) of 258 antral biopsies were positive for *H. pylori* DNA as the 501 bp amplicon was produced by nested PCR. All

nested amplicons of 501 bp were restricted into two fragments of 310 bp and 191 bp by the *Hind*III restriction enzyme (Figures 1 and 2). Although colonies grown after overnight incubation could give amplification with first round of PCR primers, nested PCR did not generate 501 bp amplicon specific to *H. pylori*. Similarly, 240 aerobic isolates could not amplify in second round PCR.

### 16S rRNA amplified rDNA restriction analysis

The isolates analyzed by using *Eco*R1 restriction endonuclease enzyme on amplicons generated by 16S rRNA specific primers fell into two groups: group A isolates could not be restricted, while group B isolates which were restricted into two fragments of 635 bp and 520 bp which were similar to *in silico* restriction of *H. pylori* J99 (Figure 3).

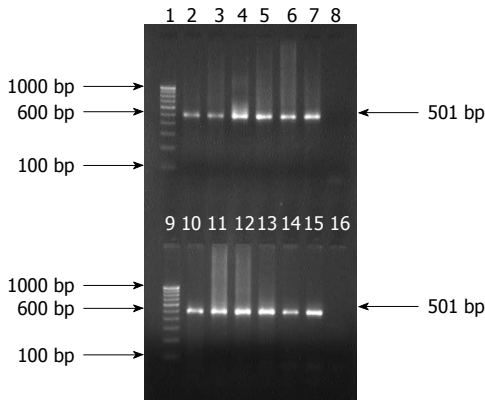
### Identification on the basis of 16S rRNA and HSP60 gene sequence

The partial nucleotide sequence of 16S rRNA of 2 isolates (GenBank accession number JQ927226 and JQ927227) from group A represented no restriction site for *Eco*R1 restriction enzyme, and comparison of the nucleotide sequences with the NCBI database showed similarity of 99% with *P. fluorescens*. Similarly, partial nucleotide sequence of unrestricted amplified *HSP60* gene (590 bp) of the isolates that grew after overnight incubation aerobically also showed 96% similarity with *P. fluorescens*.

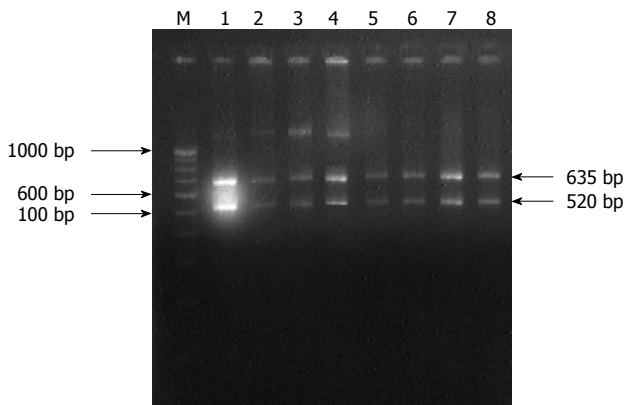
### Phylogenetic sequence analysis

The partial sequence of the 16S rRNA gene of two *P. fluorescens*-like isolated strains and sequences of 16S rRNA gene from 34 reference strains representative of the principal *Pseudomonas* phyla and 12 other bacterial pathogens were used for comparison of a phylogenetic tree. Similarly, a partial sequence of *HSP60* gene of 8 *P. fluorescens*-like isolates and sequence of *HSP60* gene of 5 *P. fluorescens* reference strains and 6 other reference strains representative of the principal *Pseudomonas* phyla and 11 other clinical pathogens were used to prepare a phylogenetic tree. To simplify the comparisons for the resulting phylogenetic tree by the UPGMA method, we named clusters based on 16S rRNA gene data, r-clusters, and those based on





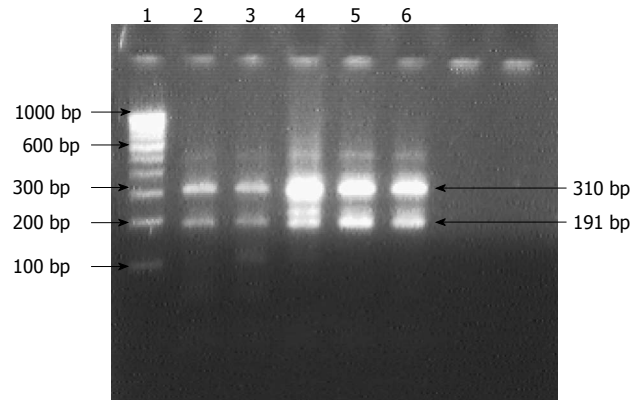
**Figure 1** Amplification of partial 501 bp *HSP60* gene with specific nested primer for *Helicobacter pylori* in antral biopsies and culture isolates. Lanes 1 and 9: Molecular marker (100 bp); Lane 2: Positive control; Lanes 3 to 7: gDNA from antral biopsies; Lanes 8 and 16: Negative control; and Lanes 10 to 15: Bacterial gDNA from culture isolates.



**Figure 3** Gel picture of amplified rDNA restriction analysis. Lane M: 100 bp molecular marker; Lanes 1 to 8: Digested 1155 bp 16S rRNA amplicon into two fragments (635 bp and 520 bp) of culture isolates of *Helicobacter pylori*.

*HSP60* data, h-clusters. The tree showed, on the basis of 16S rRNA sequences, it can be grouped clearly into three r-clusters on the basis of number of bootstraps i.e., 90, 78 and 60. Cluster r-1 represents a *fluorescens* group along with two sequences submitted to gene bank (accession number as JQ927226 and JQ927227 for RCa25 and RCa24 respectively). Cluster r-2 represents *Pseudomonas aeruginosa*, *Pseudomonas syringae*, *Pseudomonas stutzeri*, *Pseudomonas chlororaphis*, *Pseudomonas putida*, *Pseudomonas pertucinogena* group and 4 *P. fluorescens* reference strains, and r-3 cluster represents other microbes and enteric pathogens rather than non-*Pseudomonas* spp. From cluster analysis it is clear that JQ927226 and JQ927227 are related to *P. fluorescens* (Figure 4).

The tree shows that, on the basis of *HSP60* sequences, three h-clusters could be observed (bootstraps number, 99, 63 and 71); the sequences of seven isolated strains from the gastric niche could be grouped in h-cluster I along with *P. fluorescens* strains while cluster II represents non-*fluorescens* *pseudomonas* species with the exception of *P. fluorescens* F113 (CP003150). The h-cluster III grouped non-*Pseudomonas* sp. including enteric pathogens along with *H. pylori*.



**Figure 2** Electrophotograph of restriction digestion of *HSP60* gene of *Helicobacter pylori* isolates with *Hind*III. Lane 1: 100 bp molecular marker; Lanes 2 to 6: Restricted 501 bp *HSP60* gene amplicon into two fragments (310 and 191 bp) of culture isolates of *Helicobacter pylori*.

Interestingly, one strain of *P. fluorescens* RCa 24 fell into this III cluster with closeness to *Stenotrophomonas maltophilia* and *Bordetella pertussis* (Figure 5).

### ***Pseudomonas fluorescens* specific PCR**

One specific pair of primers targeting putative outer membrane protein was used to identify *P. fluorescens*. The other specific primers targeting mupirocin biosynthetic gene were used to screened out whether any *P. fluorescens*-like isolate was producing mupirocin. Genomic DNA extracted from group-A isolates and all biopsies was used as template for PCR amplification. A total of 250 (96.9%) out of 258 biopsy specimen genomic DNA samples and 240 bacterial isolates were positive for the amplification of the corresponding gene, i.e., the putative outer membrane protein gene sequences (Table 2 and Figure 6). Although putative outer membrane protein gene targeting primers were degenerate, they were unable to produce the 613 bp amplicon from *Pseudomonas aeruginosa* and *Pseudomonas putida* at similar PCR conditions. The mupirocin biosynthetic gene targeting primers were unable to produce amplification of the 722 bp or 611 bp amplicon either in primary or secondary round PCR, respectively, from any of the isolates (data not shown).

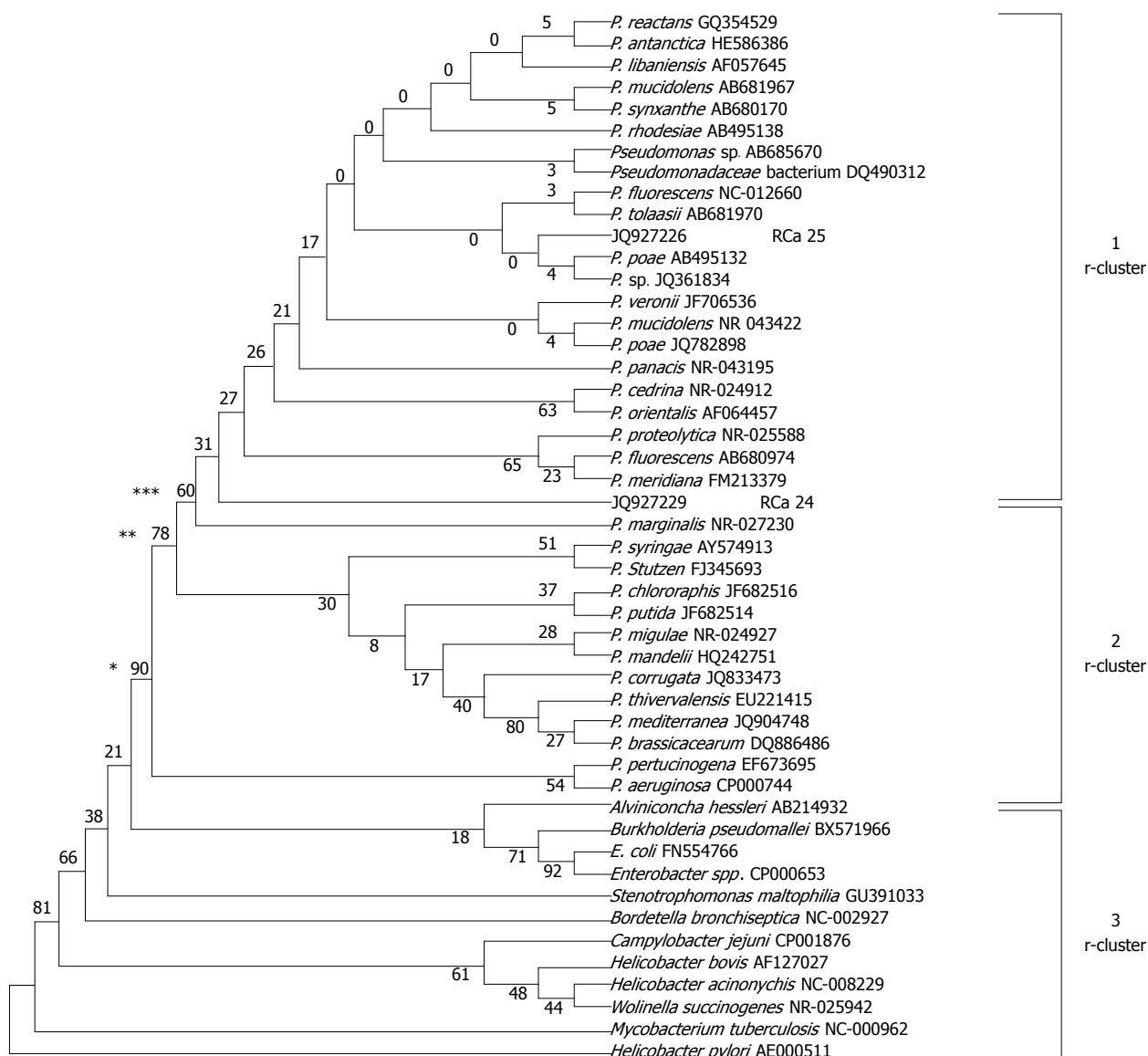
### **RAPD**

All the 71 strains tested from group-A yielded significant PCR products with RAPD primers. The strains generated approximately 3-13 well-defined bands between 150 bp to 2.5 kb sizes (9 bands on average) with each isolate yielding a unique profile of products. Cluster analysis with a RAPD-PCR based method showed that only a few isolates exhibited an identical profile. Nearly all the isolates appeared as dissimilar from each strain, but 5 strains isolated from cancer patients showed similar banding pattern at 0.0 coefficient (Figures 7 and 8).

## **DISCUSSION**

Traditionally, the human stomach has been viewed as an

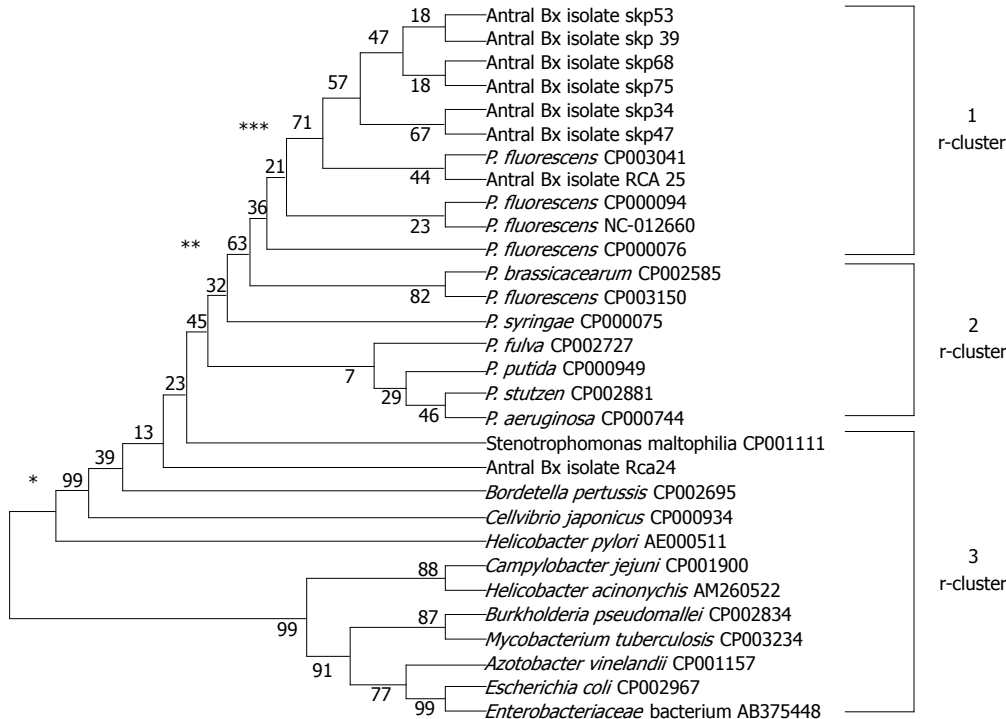




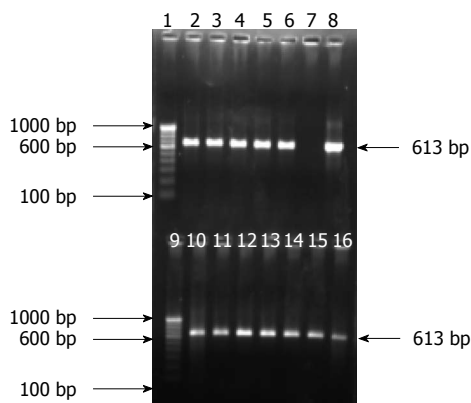
**Figure 4** Phylogenetic affiliation of the *Pseudomonas fluorescens*-like isolates ( $n = 2$ ; JQ927226 and JQ927227). The unrooted tree was generated using unweighted pair group method with arithmetic mean from evolutionary distance computed with bootstrap test of phylogeny using MEGA version 4 by aligning published sequences from Genbank of 16S rRNA genes from 34 reference strains representative of the principal *Pseudomonas* (*P.*) phyla (accession number followed by species name in parentheses). For checking relatedness with other genera, we included 16S rRNA gene sequences from GenBank of 12 bacterial pathogens namely *Stenotrophomonas maltophilia*, *Bordetella bronchiseptica*, *Wolinella succinogenes*, *Helicobacter pylori*, *Helicobacter acinonychis*, *Escherichia coli* (*E. coli*), *Enterobacter* spp., *Campylobacter jejuni*, *Burkholderia pseudomallei*, *Alviniconcha hessleri*, *Helicobacter bovis* and *Mycobacterium tuberculosis*. Branches found by maximum likelihood are labeled with asterisks: one asterisk if bootstrap values = 90%, two asterisks if = 78% and three asterisks if = 60%.

inhospitable environment for microorganisms because of its acidic environment along with several other antimicrobial factors. With the discovery of *H. pylori* and other gastric helicobacters, and subsequent insight into the mechanisms by which these organisms adapt to the gastric environment<sup>[17]</sup>, the existence of a bacterial community adapted to this human niche seems quite plausible. This is the first report of its kind showing the presence of a *P. fluorescens*-like bacterium in the human stomach. We isolated the bacteria from NUD, gastric ulcer, duodenal ulcer and gastric carcinoma patients with the belief that only *H. pylori* is associated with acid peptic diseases. However, *P. fluorescens* grew on a simple medium like Mueller-Hinton agar without an antibiotic supplement. The small white

colonies had Gram-negative slightly curved rods and produced oxidase, urease and catalase enzymes. These colonies appeared after overnight incubation in an aerobic environment. These isolates exhibited growth at 4 °C, which is one of the key characteristics of *P. fluorescens*<sup>[9]</sup>. When these isolates were exposed to acidic pH as low as pH 1.0 for 1 h, the subsequent growth of the bacteria was not affected, indicating that they were acid tolerant. However, due to deviation from the classical *H. pylori* growth characteristics, we were prompted to consider them as non-*H. pylori* and submit them for further characterization. For the purpose, *H. pylori* specific primers for amplifying *HSP60* gene-specific sequences were applied<sup>[11]</sup>. However, an approximate 600 bp sized amplicon

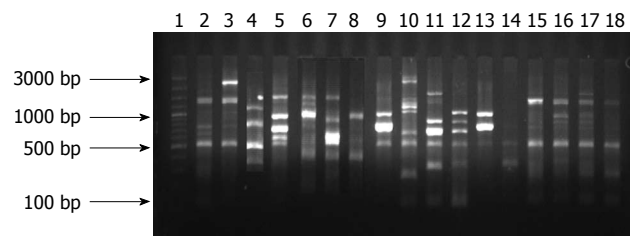


**Figure 5** Phylogenetic affiliation of the *Pseudomonas fluorescens*-like isolates ( $n = 8$ ; partial sequences of *HSP60*) on the basis of *HSP60* gene sequences. The unrooted tree was generated using unweighted pair group method with arithmetic mean from evolutionary distance computed with bootstrap test of phylogeny using MEGA version 4 by aligning published sequences from Genbank of heat shock protein (*HSP60*) genes from 5 reference strains of *Pseudomonas fluorescens* (*P. fluorescens*), 6 other reference strains representative of the principal *Pseudomonas* phyla (accession number followed by species name in parentheses). For checking relatedness with other genera, we included *HSP60* gene sequences from GenBank of 11 bacterial pathogens, namely *Helicobacter pylori*, *Helicobacter acinonychis*, *Campylobacter jejuni*, *Enterobacter* spp., *Escherichia coli*, *Bordetella pertussis*, *Burkholderia pseudomallei*, *Stenotrophomonas maltophilia*, *Cellvibrio japonicus*, *Azotobacter vinelandii* and *Mycobacterium tuberculosis*. Branches found by maximum likelihood are labeled with asterisks: one asterisk if bootstrap values = 99%, two asterisks if = 63% and three asterisks if = 71%.



**Figure 6** Amplification of putative outer membrane protein gene with specific nested primer generating 613 bp amplicon for *Pseudomonas fluorescens* of antral biopsies and culture isolates. Lanes 1 and 9: Molecular marker (100 bp); Lanes 2 to 6: gDNA from antral biopsy specimens; Lane 7: Negative control; Lane 8: Positive control; and Lanes 10 to 16: Bacterial gDNA from culture isolates.

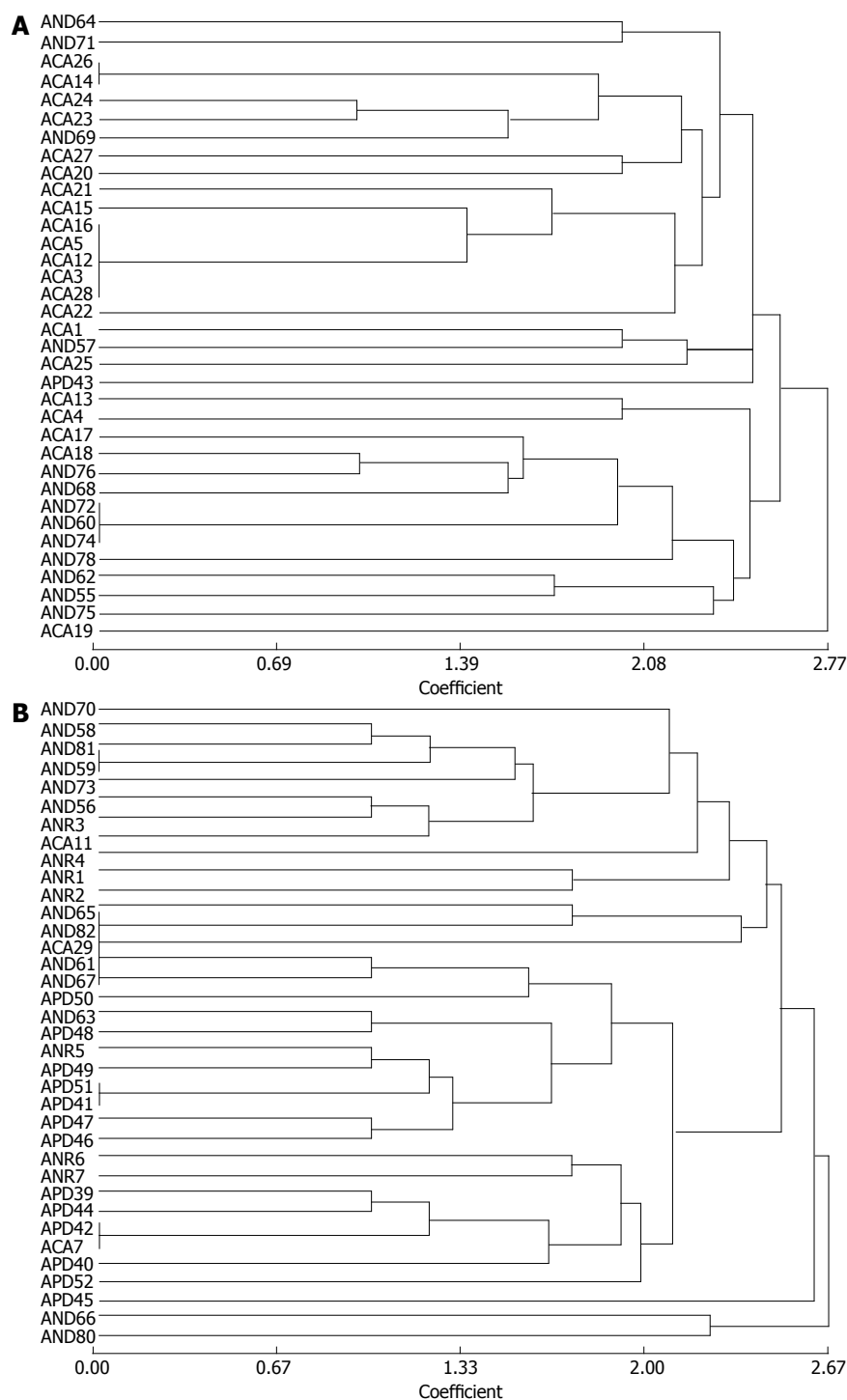
was produced by the first round of PCR, but a restriction site specific for *H. pylori* for *HindIII* enzyme was absent in these amplicons. Further, none of these isolates could yield a 501 bp amplicon for *H. pylori* by nested PCR primers. Thus, the possibility of these isolates being *H. pylori* was excluded. On the other hand, the typical *H. pylori* isolates which grew after 5-7 d of incubation yielded 501 bp



**Figure 7** Representative gel showing amplification of *Pseudomonas fluorescens* genomic sequences by randomly amplified polymorphic DNA primers. Randomly amplified polymorphic DNA (RAPD) 3' 5'-TACAGCTCG-3' and RAPD5 5'-AGCACTGCCT-3'. Lane 1: 100 bp molecular marker; Lanes 2 to 18: *Pseudomonas fluorescens* isolates isolated from stomach.

amplicon by nested PCR protocol targeting the *HSP60* gene.

Identification based on partial nucleotide sequencing of 16S rRNA of the representative isolates showed 99% sequence similarity with *γ-proteobacteria*. On the basis of enzyme restriction analysis as well as partial nucleotide sequencing of 16S rRNA, the strains isolated from the acidic environment of the stomach either could be grouped in the genus *Pseudomonas* or with a closely related new genus. Further, on the basis of blasting of partial nucleotide sequences of *HSP60* gene sequences on the NCBI gene data bank, isolated strains could be grouped with *P. fluorescens*. On the basis of h cluster based on the



**Figure 8** Dendrogram generated by unweighted pair-group method, arithmetic mean on basis of banding pattern of randomly amplified polymorphic DNA for *Pseudomonas fluorescens*. A: 1-35 out of 71 randomly selected isolates of *Pseudomonas fluorescens* from group A ( $n = 35$ ); B: 36-71 out of 71 randomly selected isolates of *Pseudomonas fluorescens* from group A ( $n = 36$ ).

UPGMA phylogenetic tree, it may be concluded that the *HSP60* gene sequence has better discriminatory power than 16S rRNA. Further, as one of the isolates could be grouped with *Stenotrophomonas maltophilia* and *Bordetella pertussis*, the possibility of the presence of bacteria other than *P. fluorescens* may not be denied.

The relatively conserved 16S rRNA gene of *P. fluorescens* has been targeted for PCR-based amplification in culture isolates, but with variable specificity<sup>[18]</sup>. Therefore,

we decided to target a conserved putative membrane-bound protein gene of *P. fluorescens*. The putative membrane-bound protein specific primer screened in the present study was found to be specific for *P. fluorescens* and it yielded an amplicon of 613 bp, confirming the isolates as being *P. fluorescens*. Mupirocin is known for its antibacterial activity and has been reported in only one strain of *P. fluorescens* (NCIMB 10586). A mupirocin-producing gene cluster could not be traced in any of the *P. fluorescens*

isolates despite confirmation by 3 genes, i.e., 16S rRNA, *HSP60* and putative membrane bound protein genes. None of our isolates showed the presence of *mupV* (mupirocin). It is quite possible that this gene might be either absent in all the strains or have too much polymorphism at the loci of primer annealing<sup>[19]</sup>. We could isolate the *P. fluorescens* strain from 93% of antral biopsies of the patients suffering from gastric diseases. These isolates were further confirmed by PCR-based amplification targeting 3 conserved genes. In DNA of antral biopsy tissue also, 98.4% of NUD and 93.9% of gastric cancer patients were found to be positive for the *P. fluorescens* DNA by nested PCR, while 95.2% of normal stomach had *P. fluorescens* and 96.9% of patients with PUD were found to have the bacterium. Thus our observation indicates a high prevalence and density of these non-*H. pylori* bacteria in the gastric mucosa of patients. Further, to ascertain that the isolated *P. fluorescens* were not contaminants, we carried out whole genome fingerprinting of the 71 randomly selected isolates by using RAPD<sup>[20,21]</sup> with two RAPD primers. Cluster analysis indicated that almost all the strains had different banding patterns with only a few exceptions, confirming that the isolates were of different clones and thus ruling out the possibility of cross contamination. Moreover, from time to time, we did specific PCR amplification for the *P. fluorescens* on DNA extracted from samples of tap water and washouts of the endoscope, which never yielded the required amplicon even by nested protocol for putative outer membrane protein.

It is interesting to mention that before the implication of *H. pylori* in acid peptic diseases and stomach cancer, Steer and Collin-Jones<sup>[4]</sup> reported that 80% of antral biopsies have *Pseudomonas* spp. There are reports showing the presence of non-*Helicobacter* bacteria in gastric biopsies of patients suffering from gastric atrophy<sup>[22,23]</sup>. In cases of reduced gastric acidity due to antacids, it also has been reported that there is presence of several other bacteria<sup>[24,26]</sup>. Two more studies based on profiling of bacterial flora by temperature gradient gel electrophoresis, 16S rRNA sequence analysis<sup>[7]</sup> and molecular analysis of the bacterial microbiota<sup>[27]</sup> have shown presence of several bacterial species including *Pseudomonas* in the stomach. This bacterium seems to be able to colonize the stomach due to its ability to produce urease enzyme. Similarly, it was reported that a non-*H. pylori* bacterium, *Ochrobactrum anthropi*, could be implicated in causation of gastritis in the Squirrel monkey<sup>[6]</sup>.

Such a high prevalence of *P. fluorescens* in human stomach raises many questions: Is it prevalent in the stomach of patients of other subcontinents and continents? Does it have any pathogenic role? Does it have some protective role? Is it simply a part of commensal flora of human stomach? There is a study from Venezuela<sup>[5]</sup> reporting that *Pseudomonas* strains may interfere with the identification of *H. pylori*. They suggested that one should not rely on rapid urease, catalase and oxidase tests for identification of *H. pylori*. However, studies are needed to observe and identify the presence of *P. fluorescens* in other parts of

the world also. With regard to its pathogenic potential, *P. fluorescens* is known as an unusual pathogen of humans. It has been reported as causing septicemia in humans, especially associated with transfusion and cancer<sup>[28-30]</sup>. Moreover, there is a report indicating that *P. fluorescens* encodes the Crohn's disease-associated *I*<sup>2</sup> sequence and T cell super antigen, thus implicating it in the pathogenesis of Crohn's disease<sup>[31]</sup>.

However, its commensal state in the stomach may be speculated strongly due to its prevalence in stomach at such a high level and density (it could be isolated in the majority of the antral biopsy). Assumptions may be made that *P. fluorescens* might be producing some antibacterial substances, such as is produced by *P. aeruginosa*, *P. aeruginosa* is known for producing 4-hydroxy-2-alkylquinoline which is inhibitory *in vitro* to *H. pylori*<sup>[32]</sup>. Moreover, one of the *P. fluorescens* strains is already known for production of mupirocin which is very effective against methicillin-resistant *Staphylococcus aureus*. This bacterium has been stated to have a probiotic role in the gills of fish<sup>[33]</sup>. Furthermore, low isolation of *H. pylori* and fewer incidences of acid peptic diseases including gastric cancer in North Indians may also be speculated on the basis of the probiotic activity of *P. fluorescens* in the stomach. In addition, there are reports of the unique property of *P. fluorescens* to inhibit the growth of other bacteria, fungi and nematodes causing plant pathology<sup>[34-40]</sup>.

In view of the suggestions made by previous studies<sup>[41-43]</sup>, there is a strong need to explore the exact role of *H. pylori* in stomach diseases because the commensal role of *H. pylori* cannot be rejected outright. In a similar way, the observations made in the present study strongly indicate that further exploration of the different aspects of associations of *P. fluorescens* with human disease and health should be carried out. The pathogenic potential may be explored in animal models like gerbils.

This study concludes that *P. fluorescens* is as common as *H. pylori* in the stomach of humans. Colonies that appeared on enriched BHI agar after 72 h of strictly micro-aerophilic incubation were *H. pylori* while those growing faster in an aerobic atmosphere were different. These different growths could be identified as *P. fluorescens*. The activity of *P. fluorescens* in the stomach may be speculated to be either pathogenic or probiotic.

## COMMENTS

### Background

Although *Helicobacter pylori* (*H. pylori*) has been implicated in acid peptic diseases along with stomach cancer, there are reports indicating the presence of several other bacterial species in the stomach. Antral biopsies from North Indian subjects frequently yielded a bacterial growth on selective, non-enriched simple medium in an aerobic environment at 37 °C of small, low convex, and pinhead-size translucent colonies. Presence of these types of growth provoked questions about its characterization and its status: whether it was a contaminant from the environment during antral biopsy collection.

### Research frontiers

The big question to be answered is further characterization of these isolates and to ensure that they are actual colonizers of the stomach. Do these acid tolerant isolates have pathogenic potential if they are real colonizers?



## Innovations and breakthroughs

*Pseudomonas fluorescens* (*P. fluorescens*)-like bacteria colonize the stomach quite frequently of North Indian patients at high density, as polymerase chain reaction (PCR)-based detection and isolation rates were both comparable. In contrast, the density of *H. pylori* seems to be quite low as nested PCR-based detection is significantly high as compared to the isolation rate of the bacterium. *P. fluorescens* isolates are urease producers and acid tolerant. Although the mupirocin gene could not be detected in any of the *P. fluorescens* isolates, the probiotic (inhibitory to *H. pylori*) role of the bacterium in the stomach may be speculated.

## Applications

The potential of *P. fluorescens* as a probiotic may be explored because despite very high prevalence of *H. pylori* in India the incidences of acid peptic diseases and stomach cancer are quite low.

## Terminology

Nested polymerase chain reaction is a modified technique of PCR intended to increase sensitivity and specificity of primer and to reduce the contamination in amplicons due to the amplification of undesired primer annealing sites. Phylogenetics is the study of evolutionary relations among groups of organisms such as strains or species, which are based on molecular sequencing data matrices. Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit to the host, e.g., lactic acid bacteria.

## Peer review

The study is well carried out from the methodological perspective. This manuscript characterized the bacterial isolates growing aerobically from antral biopsies were *P. fluorescens*, which was the acid tolerant bacteria other than *H. pylori*. On the basis of 16S rRNA and HSP60 sequence and from phylogenetic sequence analysis these organisms were closely related to *P. fluorescens*. The authors also reconfirmed this unknown bacterium as *P. fluorescens* by PCR positivity of *P. fluorescens* specific conserved putative outer membrane protein gene. Finally, the authors concluded that *P. fluorescens* is as common as *H. pylori* in the human stomach.

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## Long interspersed nuclear element ORF-1 protein promotes proliferation and resistance to chemotherapy in hepatocellular carcinoma

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long interspersed nuclear element-1 ORF-1 protein [human long interspersed nuclear element-1 (LINE-1), ORF-1p] in chemotherapeutic drug resistance and cell proliferation regulation in hepatocellular carcinoma (HCC) cells.

**METHODS:** MTT assays were performed to identify the effect of the chemotherapeutic drug toxicity on HepG2 cells. Cell proliferation inhibition and the IC<sub>50</sub> were calculated by the Origin 8.0 software. Western blotting assays were performed to investigate whether LINE-1 ORF-1p modulates the expression of some important genes, including *p53*, *p27*, *p15*, *Bcl-2*, *mdr*, and *p-gp*. To corroborate the proliferation and anchor-independent growth results, the HepG2 cells were analyzed by flow cytometry to investigate the effect of LINE-1 ORF-1p on the apoptosis regulation.

**RESULTS:** LINE-1 ORF-1p contributed to the resistance to several chemotherapeutic drugs (cisplatin and epirubicin) in HepG2 cells. The IC<sub>50</sub> of the epirubicin and cisplatin increased from 36.04 nmol/L to 59.11 nmol/L or from 37.94 nmol/L to 119.32 nmol/L. Repression of LINE-1 ORF-1p expression by the siRNA could markedly enhance the response of HepG2 cells to the epirubicin and cisplatin. The IC<sub>50</sub> correspondingly decreased from 28.06 nmol/L to 3.83 nmol/L or from 32.04 nmol/L to 2.89 nmol/L. Interestingly, down-regulation of LINE-1 ORF-1p level by siRNA could promote the response of HepG2 cells to the paclitaxel. The IC<sub>50</sub> decreased from 35.90 nmol/L to 7.36 nmol/L. However, overexpression of LINE-1 ORF-1p did not modulate the paclitaxel toxicity in HepG2 cells. Further Western blotting revealed that LINE-1 ORF-1p enhanced *mdr* and *p-gp* gene expression. As a protein arrested in the nucleus, LINE-1 ORF-1p may function through modulating transcriptional activity of some important transcription factors. Indeed, LINE-1 ORF-1p promoted HepG2 cell prolifera-

### Abstract

**AIM:** To clarify the specific roles and mechanisms of

tion, anchor-independent growth and protected the cells against apoptosis through modulating the expression of *p15*, *p21*, *p53*, and *Bcl-2* genes.

**CONCLUSION:** LINE-1 ORF-1p promotes HepG2 cell proliferation and plays an important role in the resistance of chemotherapeutic drugs. By establishing novel roles and defining the mechanisms of LINE-1 ORF-1p in HCC chemotherapeutic drug resistance and cell proliferation regulation, this study indicates that LINE-1 ORF-1p is a potential target for overcoming HCC chemotherapeutic resistance.

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**Key words:** Long interspersed nuclear element-1 ORF-1 protein; Hepatocellular carcinoma; Chemotherapeutic drugs; Multi-drug resistance

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## INTRODUCTION

Hepatocellular carcinoma (HCC) which is the most common tumor worldwide, and the third most common cause of cancer death, is often accompanied by a poor prognosis<sup>[1]</sup>. The majority of HCC patients are unresectable at the time of first diagnosis, and even in patients who are suitable for surgery, the risk of recurrence is high. Consequently, chemotherapy is an important alternative strategy for most HCC patients<sup>[2]</sup>. Cisplatin, paclitaxel and epirubicin are common chemotherapeutic agents used in the treatment of HCC. However, chemotherapy is often ineffective in HCC patients due to potent multidrug resistance (MDR)<sup>[3]</sup>. Many studies have indicated that alterations in target gene expression are correlated with drug resistance and may provide a new strategy for sensitizing cancer cells to chemotherapeutic drugs and to reverse MDR in cancer cells.

The human long interspersed nuclear element-1 (*LINE-1*) gene encodes two proteins: LINE-1 ORF-1p and LINE-1 ORF-2p<sup>[4]</sup>. It is reported that LINE-1 ORF-2p is mainly involved in the retrotransposition process through its endonuclease activity<sup>[4]</sup>. In human cancer cells, LINE-1 ORF-2p can be regulated by LINE-1 ORF-1p<sup>[4,5]</sup>. The expression of the LINE-1 ORF-1 protein is almost 1000-fold greater than the LINE-1 ORF-2 protein<sup>[5]</sup>, which indicates that LINE-1 ORF-1p has many roles. Our previous results suggested that LINE-1 ORF-1p enhances the proliferation of several cancer cell lines<sup>[6]</sup>. LINE-1 ORF-1p also increased the

proliferation of hepatocellular carcinoma cells Bel-7402, SMMC-7721, HepG2, and LO2 cells and was associated with a significant risk of HCC progression<sup>[7]</sup>. However, the detailed mechanisms and roles of LINE-1 ORF-1p in HCC development are still largely unknown.

Based on our previous study<sup>[7]</sup>, we hypothesized that LINE-1 ORF-1p might also be involved in HCC development and chemotherapy resistance regulation. To address this hypothesis, we performed MTT assays and found that LINE-1 ORF-1p protects HepG2 cells against chemotherapy. More importantly, we also found that LINE-1 ORF-1p enhances HepG2 cell proliferation through the modulation of genes involved in cell proliferation and apoptosis such as *p53* and *Bcl-2*.

## MATERIALS AND METHODS

### Plasmids

The full length of LINE-1 ORF-1p cDNA was cloned into a pcDNA3.1 vector linked with fludarabine + high-dose cytarabine + G-CSF (FLAG) at the amino terminus by polymerase chain reaction using a cDNA library as the template<sup>[6,7]</sup>. The siRNA targeted to LINE-1 ORF-1p was cloned into the psilencer-2.1-U6 (neo) vector (Ambion). The sequence of siRNA is AAGGAGGTGCACTATA-AGAAC<sup>[6,7]</sup>. The luciferase reporters, Luc-p21 (containing p53 binding element), Luc-Smad4 binding element (SBE), Luc-SP1 and Luc-androgen response element (ARE) were gifts from Dr. Cui JJ and Yang YT.

### Antibodies

Antibodies against *p27*, *p53*, *Bcl-2*, and *p15* were from Santa Cruz Biotechnology Inc., United States, and antibodies against MDR, p-gp and GAPDH were from Sigma-Aldrich, St. Louis, United States. The antibody against LINE-1 ORF-1p was described previously<sup>[6,7]</sup>. Polyclonal anti-rabbit IgG was from Qiagen, Beijing, China.

### Chemotherapeutic agents and cell culture

Epirubicin (Pfizer, NY, United States), Cisplatin (QILU Pharmaceutical Co., Jinan, China), paclitaxel (Roche, Basel, Swiss), Lipofectamine 2000 (Invitrogen, Carlsbad, CA, United States) and other agents (*Amersham Biosciences*, Piscataway, NJ, United States) were used in this study. HepG2 cells (American Type Culture Collection, ATCC) were cultured in DMEM (GIBCO, Grand Island, NY, United States) medium containing 10% FBS.

### Cell proliferation assays

HepG2 cells were transfected with FLAG-LINE-1 ORF-1p or siRNA plasmid, and cultured for 24 h at 37 °C and 5% CO<sub>2</sub>. Then, 10 µL CCK-8 reagent was added to each well and cultured at 37 °C and 5% CO<sub>2</sub> for 4 h. The absorbance of the inhibition rate was measured using a multifunctional microplate reader at 490 nm. The inhibition rate = (*A* control group - *A* administration group) / (*A* control group - *A* blank group) × 100%. The assays were performed three times with similar results.



**Table 1** Effect of long interspersed nuclear element ORF-1 protein on the cytotoxic activity of chemotherapeutic drugs *in vitro*

Drugs	IC <sub>50</sub> (nmol/L)			
	FLAG	FLAG-ORF-1p	Control	siRNA
Epirubicin	36.04 ± 4.48	59.11 ± 3.37	28.06 ± 5.31	3.84 ± 0.48
Cisplatin	37.94 ± 2.90	119.32 ± 21.99	32.04 ± 3.71	2.89 ± 0.44
Paclitaxel	27.01 ± 3.91	29.01 ± 2.95	35.90 ± 7.03	7.36 ± 0.69

FLAG: Fludarabine + high-dose cytarabine + G-CSF; siRNA: Small interfering RNA.

### Anchorage-independent growth assay

HepG2 cells which stably expressed FLAG-LINE-1 ORF-1p or siRNA were placed in 6-well plates, with a bottom layer of 0.7% low-melting-temperature agar in DMEM and a top layer of 0.25% agar in DMEM. Colonies were scored after 3 wk of growth. The assays were performed in three independent experiments with similar results.

### Luciferase assay

HepG2 cells were seeded in 24-well plates. After 24 h, the cells were transfected with FLAG-LINE-1 ORF-1p and the indicated reporter gene using Lipofectamine 2000 (Invitrogen). Twenty-four hours later, the cells were harvested and analyzed for luciferase following the manual protocol (Promega Corp., Madison, WI, United States).

### Flow cytometry and apoptosis analysis

Assays were performed following the protocol provided by the apoptosis assay kit (Qiagen, Beijing, China). In brief, cells were treated with dehydrated alcohol for flow cytometry analysis. Before analysis, cells were treated with 0.5% RNase at 65 °C for 30 min. The assays were performed three times with similar results.

### Cell transfection and stable transfection

Plasmids were transfected into HepG2 cells using Lipofectamine 2000 (Invitrogen). Forty-eight hours later, transfected cells were cultured in 500 µg/mL G418 (Invitrogen) for approximately 2 mo. Individual clones were screened by Western blotting using anti-FLAG or anti-LINE-1 ORF-1p antibodies. Similar results were observed with stable transfection or transient transfection with individual clones or pool clones.

### Immunoblotting analysis

Total protein in the samples was measured by SDS-PAGE and trans-printed to a nitrocellulose (NC) membrane. The NC membranes were blocked with 5% BSA in TBST buffer and incubated with the antibodies. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies after washing with TBST buffer. Membranes were visualized using the appropriate kit (Qiagen).

## RESULTS

### LINE ORF-1 protein modulates the cytotoxic effects of chemotherapeutic agents

To determine whether LINE-1 ORF-1p modulates the cytotoxic effects of chemotherapeutic agents, MTT assays were performed. The results showed that overexpression of LINE-1 ORF-1p significantly reduced the cytotoxicity of epirubicin and cisplatin on HepG2 cells (Figure 1) and the corresponding IC<sub>50</sub> values increased significantly (Table 1). Reduction of LINE-1 ORF-1p expression by siRNA markedly promoted the sensitivity of HepG2 cells to epirubicin and cisplatin (Figure 1). The IC<sub>50</sub> values correspondingly decreased significantly (Table 1).

Interestingly, our data also showed that neither overexpression of LINE-1 ORF-1p plasmids nor the empty vector protected HepG2 cells from the cytotoxicity of paclitaxel (Figure 1E). Knockdown of LINE-1 ORF-1p increased the cytotoxic effect of paclitaxel on HepG2 cells (Figure 1F). The IC<sub>50</sub> values correspondingly decreased from 35.90 nmol/L to 7.36 nmol/L (Table 1). Taken together, these results suggest that LINE-1 ORF-1p mediates chemotherapeutic drug resistance in HepG2 cells.

### LINE ORF-1 protein promotes HepG2 cell proliferation

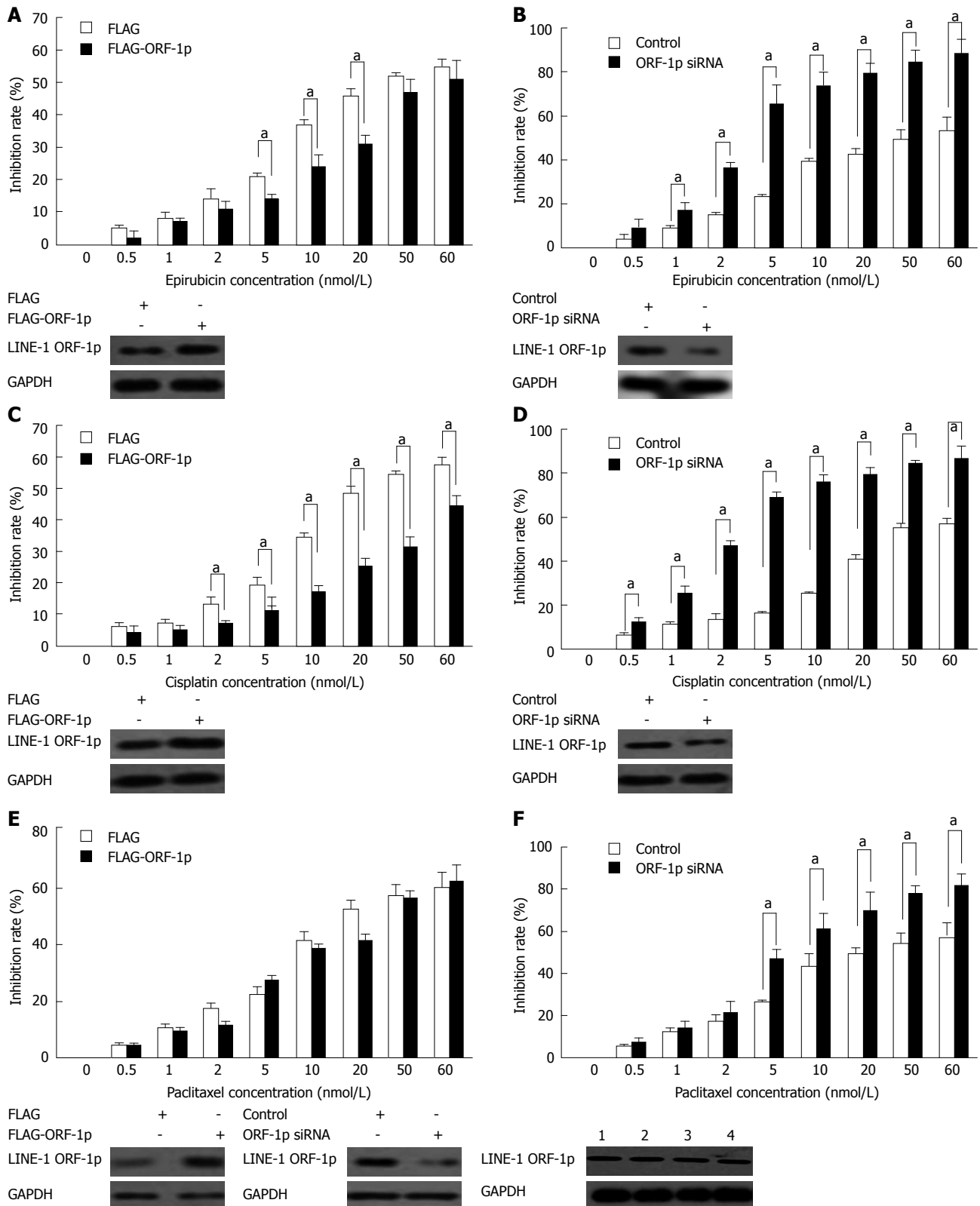
To determine whether LINE-1 ORF-1p affected HepG2 cell proliferation, MTT assays were performed. HepG2 cells transfected with FLAG-LINE-1 ORF-1p had an increased proliferation rate compared with those transfected with FLAG empty vector (Figure 2A); whereas cells transfected with LINE-1 ORF-1p siRNA, grew more slowly than those transfected with the control siRNA (Figure 2B). In addition, LINE-1 ORF-1p also promoted HCC SMMC-7721 (Figure 2C and D) and BEL-7402 cell growth (Figure 2E and F). These results indicated that LINE-1 ORF-1p affects HCC cell proliferation.

### LINE ORF-1 protein protects HepG2 cells against apoptosis

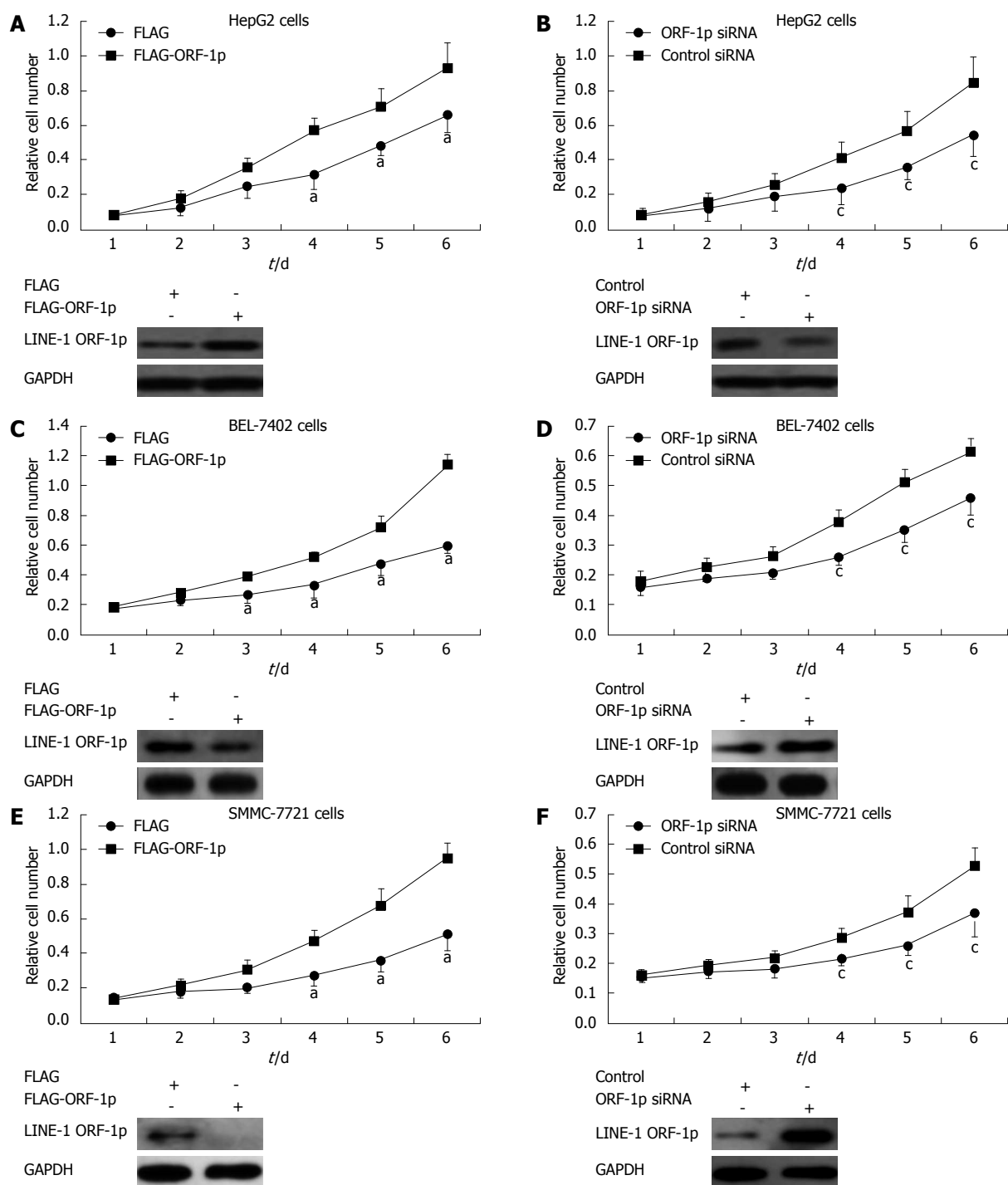
To investigate the role of LINE-1 ORF-1p in HepG2 cell apoptosis, apoptosis assays were performed. Overexpression of LINE-1 ORF-1p protected HepG2 cells from apoptosis (Figure 3), whereas knockdown of LINE-1 ORF-1p significantly promoted the apoptosis of HepG2 cells (Figure 3).

### LINE ORF-1 protein increases HepG2 anchorage-independent growth

The effects of LINE-1 ORF-1p on anchorage-independent growth were tested in stable HepG2 cell lines expressing either FLAG-LINE-1 ORF-1p or siRNA. Overexpression of LINE-1 ORF-1p significantly increased the anchorage-independent growth of HepG2 cells (Figure 3); whereas knockdown of endogenous LINE-1 ORF-1p decreased anchorage-independent growth of HepG2 cells (Figure 3). These results suggest that LINE-1 ORF-1p also strongly promotes HCC cell growth.



**Figure 1** Effect of long interspersed nuclear element ORF-1 protein on epirubicin, cisplatin and paclitaxel cytotoxicity. HepG2 cells were treated with the indicated amounts of epirubicin (A, B), cisplatin (C, D) or paclitaxel (E, F): 0 nmol/L, 0.5 nmol/L, 1 nmol/L, 2 nmol/L, 5 nmol/L, 10 nmol/L, 20 nmol/L, 50 nmol/L, 60 nmol/L. A: HepG2 cells were transfected with the fludarabine + high-dose cytarabine + G-CSF human long interspersed nuclear element-1 (FLAG-LINE-1) ORF-1p expression vector, or the empty vector; B: HepG2 cells were transfected with the control siRNA vector or the LINE-1 ORF-1p siRNA vector; C: HepG2 cells were transfected with the FLAG-LINE-1 ORF-1p expression vector, or the empty vector; D: HepG2 cells were transfected with the control siRNA vector or the LINE-1 ORF-1p siRNA vector; E: HepG2 cells were transfected with the FLAG-LINE-1 ORF-1p expression vector, or the empty vector; F: HepG2 cells were transfected with the control siRNA vector or the LINE-1 ORF-1p siRNA vector. The assays were performed three times with similar results. \* $P < 0.05$  vs control. 1: Control; 2: Epirubicin; 3: Cisplatin; 4: Paclitaxel. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.



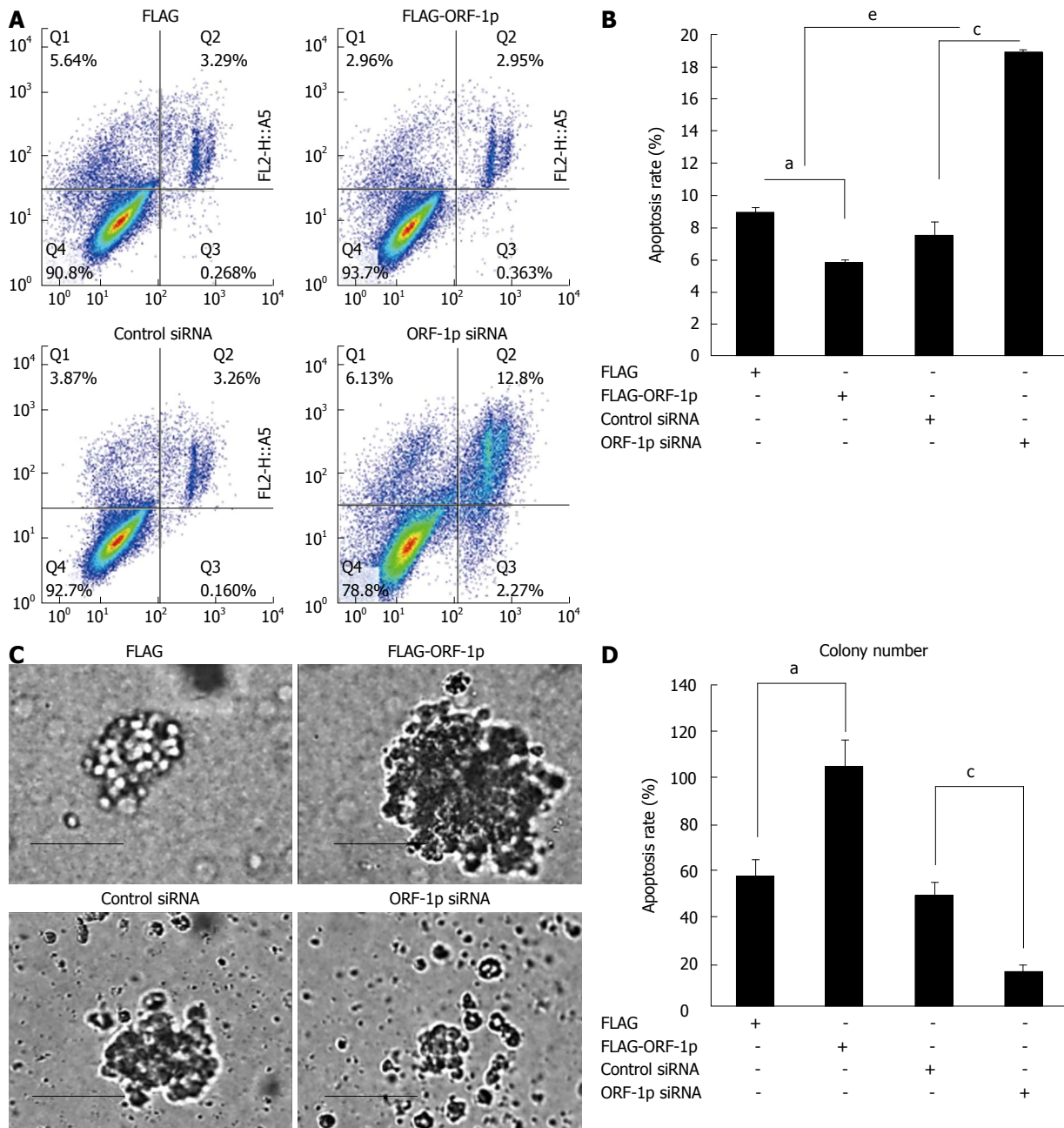
**Figure 2** Long interspersed nuclear element ORF-1 protein promotes the proliferation of hepatocellular carcinoma cells. A: HepG2 cells were stably transfected with the fludarabine + high-dose cytarabine + G-CSF human long interspersed nuclear element-1 (FLAG-LINE-1) ORF-1p or the empty vector; B: HepG2 cells were stably transfected with the control siRNA or LINE-1 ORF-1p siRNA; C: BEL-7402 cells were stably transfected with the FLAG-LINE-1 ORF-1p or the empty vector; D: BEL-7402 cells were stably transfected with the control siRNA or LINE-1 ORF-1p siRNA; E: SMMC-7721 cells were stably transfected with the FLAG-LINE-1 ORF-1p or the empty vector; F: SMMC-7721 cells were stably transfected with the control siRNA or LINE-1 ORF-1p siRNA. The relative cell numbers ( $A_{490\text{ nm}}$ ) were determined by MTT assays, relative cell numbers (A, B) with mean  $\pm$  SD ( $n = 3$ ).  $^aP < 0.05$  vs FLAG;  $^cP < 0.05$  vs control siRNA. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

### LINE ORF-1 protein regulates the reporter gene activities of p21, SBE and Sp1

To determine the mechanism of LINE-1 ORF1-p, luciferase assays were performed. The results showed that overexpression of LINE-1 ORF-1p decreased Luc-p21, Luc-SBE and Luc-Sp1 activity. Knock-down of LINE-1 ORF-

1p expression enhanced the activities of Luc-p21 (Figure 4A), Luc-SBE (Figure 4B) and Luc-Sp1 (Figure 4C).

In addition, overexpression of LINE-1 ORF-1p promoted the transcription of Luc-ARE reporter (Figure 4D). Down-regulation of LINE-1 ORF1-p expression reduced the activity of Luc-ARE (Figure 4D). Taken to-



**Figure 3** Long interspersed nuclear element ORF-1 protein promotes apoptosis and anchor-independent growth of HepG2 cells. HepG2 cells were transfected with the empty vector or f ludarabine + high-dose cytarabine + G-CSF human long interspersed nuclear element-1 (FLAG-LINE-1) ORF-1p, or with the control siRNA or LINE-1 ORF-1p siRNA. A, B: The rates of apoptosis are shown in the figures (A). The assays were performed three times with similar results (B); C, D: Colony numbers shown in the photographs (C), or mean  $\pm$  SD ( $n = 3$ ) of triplicate independent experiments (D). <sup>a</sup> $P < 0.05$  vs FLAG; <sup>b</sup> $P < 0.05$  vs control siRNA; <sup>c</sup> $P < 0.05$  vs FLAG-LINE-1 ORF-1p. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

gether, these results suggest that LINE-1 ORF-1p may regulate cell proliferation by modulating the transcription activities of these genes

#### LINE ORF-1 protein modulates the expression of genes involved in cell proliferation and apoptosis

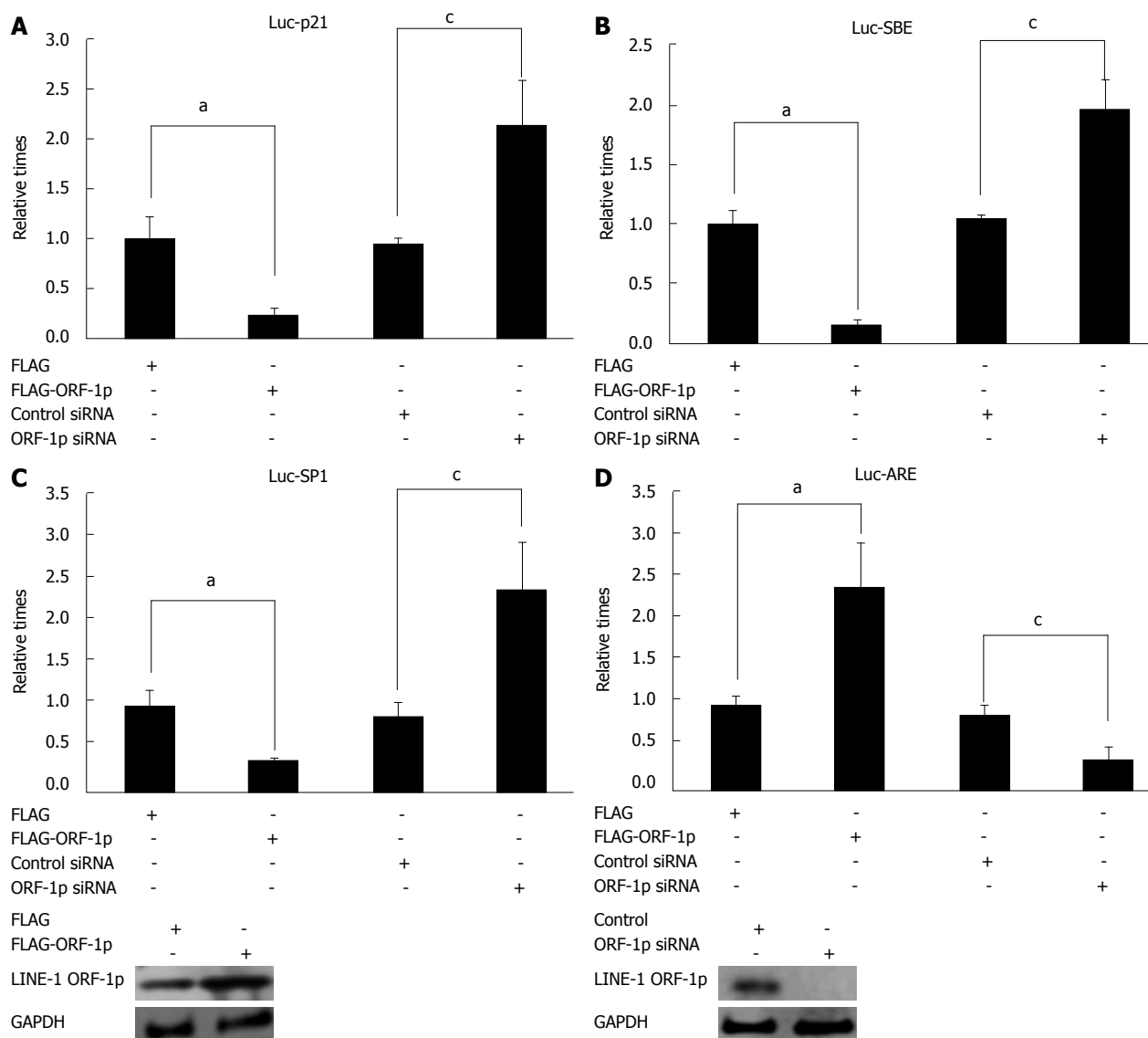
To corroborate the results of the luciferase assay, it is necessary to investigate whether LINE-1 ORF-1p regulates the protein levels of the genes related to cell proliferation and apoptosis. The results showed that overexpression of LINE-1 ORF-1p decreased the expression of *p15*, *p53* and *p27* genes (Figure 5A) and increased the

expression of Bcl-2 (Figure 5A). However, reduction of LINE-1 ORF-1p expression with LINE-1 ORF-1p siRNA increased the protein level of *p15*, *p53* and *p27* (Figure 5B), and reduced the protein level of Bcl-2 (Figure 5B). These findings indicated that LINE-1 ORF-1p promotes cell proliferation by regulating the expression of these genes.

#### LINE ORF-1 protein enhances MDR and p-gp gene expression

In order to investigate the mechanism of LINE-1 ORF-1p-mediated drug resistance, the effects of LINE-1





**Figure 4** Long interspersed nuclear element ORF-1 protein regulates the transcription of Luciferase reporters. HepG2 cells were transfected with the fludrabin + high-dose cytarabine + G-CSF human long interspersed nuclear element-1 (FLAG-LINE-1) ORF-1p or FLAG empty vector, LINE-1 ORF-1p siRNA or control siRNA. HepG2 cells were co-transfected with Luc-p21 (A), Luc-SBE (B), Luc-SP1 (C), or Luc-ARE (D). The assays were performed three times with similar results. <sup>a</sup>*P* < 0.05 vs FLAG; <sup>b</sup>*P* < 0.05 vs control siRNA. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

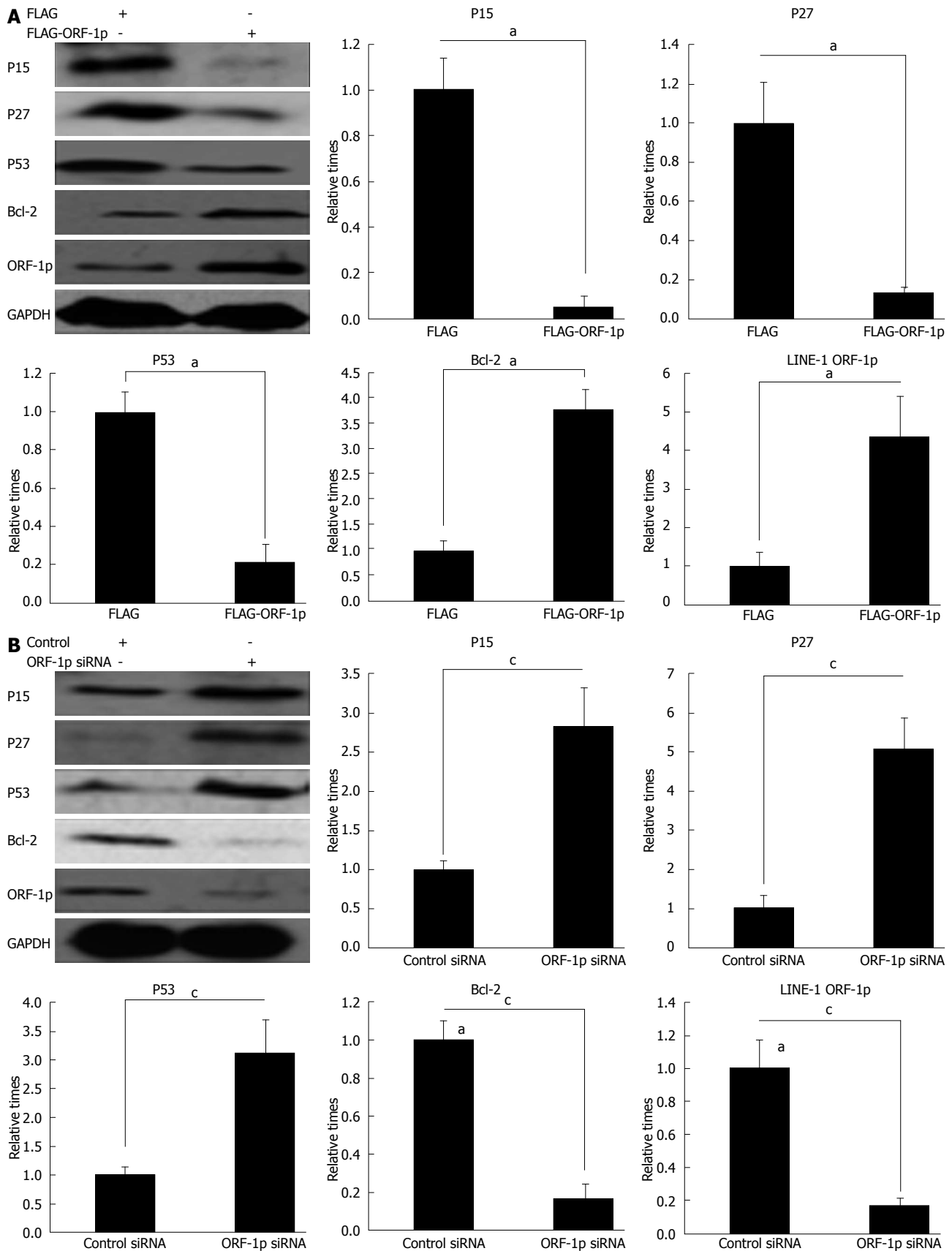
ORF-1p on the expression of genes involved in drug resistance were examined. The results showed that over-expression of LINE-1 ORF1-p increased MDR and p-gp protein levels (Figure 6A). Repression of LINE-1 ORF-1p expression through siRNA strongly reduced MDR and p-gp protein levels (Figure 6B). These results indicated that LINE-1 ORF-1p mediates drug resistance by modulating the expression of MDR and p-gp.

#### LINE ORF-1 protein is expressed in clinical specimens and HepG2 cells

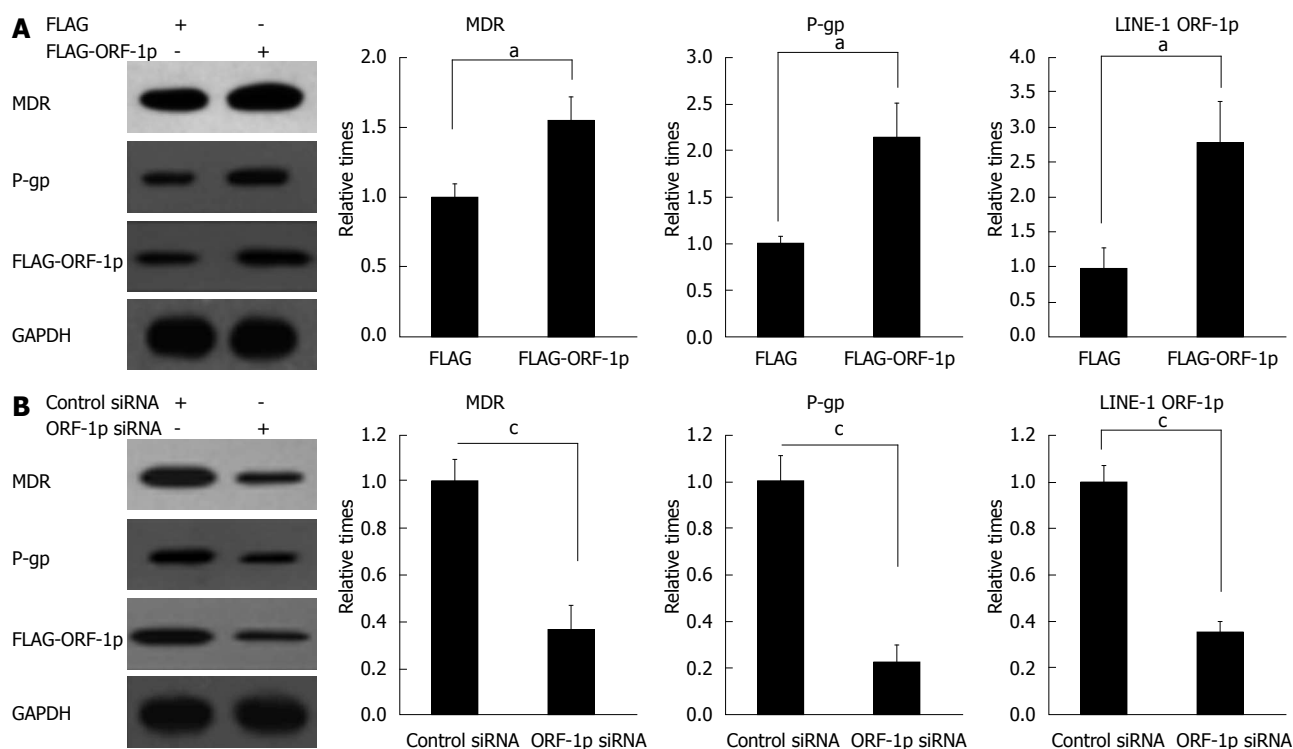
Next, we used immunohistochemistry and immunoblot assays to detect LINE-1 ORF-1p protein expression in clinical specimens and the HepG2 cell line. In most cases, the HepG2 cell line and liver tumors expressed high protein levels of LINE-1 ORF-1p (Figure 7).

## DISCUSSION

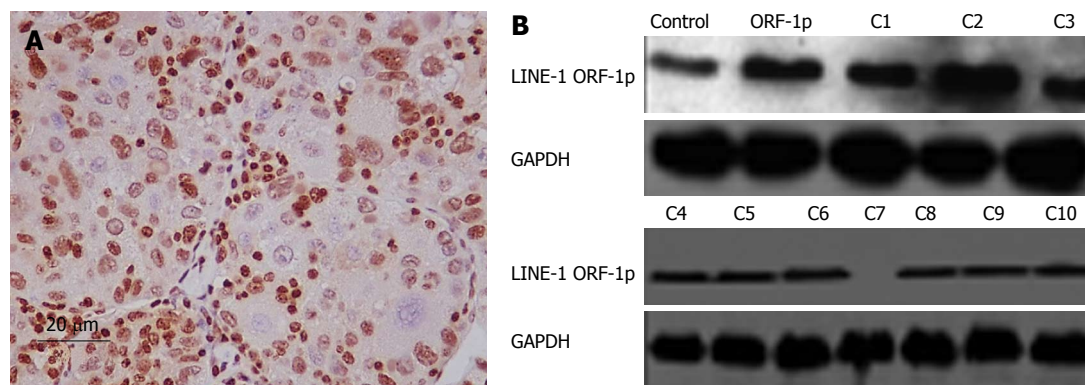
In this study, we provide evidence of the novel roles of LINE-1 ORF-1p in chemotherapeutic drug response and proliferation of HepG2 cells. The results demonstrated that over-expression of LINE-1 ORF-1p significantly inhibited the cytotoxic effects of epirubicin and cisplatin on HepG2 cells. However, knockdown of LINE-1 ORF-1p with epirubicin, cisplatin or paclitaxel had a synergistic effect on HepG2 cells. Moreover, LINE-1 ORF-1p regulated the proliferation and anchor-independent growth of HepG2 cells. In addition, LINE-1 ORF-1p repressed Luc-SBE, Luc-SP1, and Luc-p21 transcription, and enhanced Luc-ARE transcription. More importantly, LINE-1 ORF-1p modulated the expression of important genes: *p15*, *p27*, *p53* and *Bcl-2*, which are related to



**Figure 5** Long interspersed nuclear element ORF-1 protein modulates the expression of genes related to cell proliferation and apoptosis regulation. **A:** HepG2 cells were transfected with the fludauridine + high-dose cytarabine + G-CSF human long interspersed nuclear element-1 (FLAG-LINE-1) ORF-1p vector, or the empty vector. The assays were performed three times with similar results. <sup>a</sup> $P < 0.05$  vs FLAG; **B:** HepG2 cells were transfected with control siRNA or LINE-1 ORF-1p siRNA. The bar graph shows the relative protein expression level of each protein compared with GAPDH. The assays were performed three times with similar results. <sup>a</sup> $P < 0.05$  vs FLAG; <sup>c</sup> $P < 0.05$  vs control siRNA. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.



**Figure 6** Long interspersed nuclear element ORF-1 protein modulates the expression of genes related to drug resistance. A: HepG2 cells were transfected with the fludarabine + high-dose cytarabine + G-CSF human long interspersed nuclear element-1 (FLAG-LINE-1) ORF-1p vector, or the empty vector; B: HepG2 cells were transfected with control siRNA or LINE-1 ORF-1p siRNA. The bar graph shows the relative protein expression level of each protein compared with GAPDH. The assays were performed three times with similar results. <sup>a</sup>*P* < 0.05 vs FLAG; <sup>c</sup>*P* < 0.05 vs control siRNA. MDR: Multidrug resistance; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.



**Figure 7** Long interspersed nuclear element ORF-1 protein expressed in clinical specimens and HepG2 cells. Representative immunohistochemical staining (A) and immunoblots (B) of human long interspersed nuclear element-1 (LINE-1) ORF-1p proteins in HepG2 cells, which were stably transfected with empty vector or LINE-1 expression vector, and in human cancerous liver tissues (C1-C10). Original magnification:  $\times 40$ . GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

HepG2 cell proliferation and apoptosis. Finally, LINE-1 ORF-1p also affected *mdr* and *p-gp* gene expression, which is related to chemotherapeutic drug resistance in cancer cells.

LINE-1 comprises at least 17% of the human genome sequence and may significantly affect human genome stability and chromosome structure after de-methylation. LINE-1 displays a silenced state in normal adult tissues with high methylation<sup>[8]</sup>. It is activated by the de-methylation mechanism, and then frequently retrotransposes<sup>[9]</sup>. Frequent transposition of LINE-1 may lead to significant changes in the genome, eventually inducing tumori-

genesis<sup>[10]</sup>. It was reported that genome hypomethylation was significantly increased in patients with HCC. The levels of serum LINE-1 hypomethylation at initial presentation correlated significantly with tumor size, tumor number and alpha-fetoprotein level. Moreover, high serum LINE-1 hypomethylation correlates significantly with poor survival<sup>[11,12]</sup>. Therefore, it is thought that over-expression of LINE-1-associated protein promotes the progression of HCC and affects the outcome of systemic chemotherapy.

Our previous studies indicated that LINE-1 ORF-1p was involved in the regulation of transformation, devel-

opment and proliferation of several cancer cells<sup>[13,14]</sup>. We also established several tumor cell models of LINE-1 ORF-1p in human lung cancer, gastric cancer and breast cancer<sup>[15]</sup>. It is reported that LINE-1 ORF-1p interacts with its own DNA<sup>[16]</sup>. Thus, LINE-1 ORF-1p may regulate important transcription factor activity by changing the structure of chromatin, and in turn promote the growth of cancer cells by modulating the expression of genes related to proliferation, cell cycling and apoptosis regulation<sup>[17-19]</sup>. LINE-1 ORF-1p may play a role in the regulation of cancer cell proliferation and chemotherapy resistance through various mechanisms.

In this study, we found that overexpression of LINE-1 ORF-1p significantly inhibited the cytotoxic effect of epirubicin and cisplatin on HepG2 cells, without affecting the role of paclitaxel, these results were expected. LINE-1 ORF-1p siRNA with epirubicin, cisplatin or paclitaxel had a synergistic effect on HepG2 cells. In HCC, the rapid growth of cells is caused by high levels of DNA replication and expression, and a high rate of metabolism. In addition, in HCC cells, chromatin is generally unfolded and not agglutinated<sup>[17-19]</sup>. Therefore, the DNA is more vulnerable than in normal somatic cells<sup>[12]</sup>. Some chemotherapeutic agents such as cisplatin and epirubicin target bio-macromolecules, such as DNA, and are naturally cytotoxic to HepG2 cells<sup>[12]</sup>. While LINE-1 ORF-1p binds to DNA, it is likely to play a protective role and act as a barrier to DNA. It is possible to protect DNA against the cytotoxic effects of epirubicin and cisplatin. Our previous study showed that LINE-1 ORF-1p promotes the cell cycling program. These previous results also confirmed the conclusions in this study. In addition, LINE-1 ORF-1p enhanced the protein level of *mdr* and *p-gp* genes<sup>[20-22]</sup>. Thus, LINE-1 ORF-1p also modulates the genes involved in multi-drug resistance.

We then found that LINE-1 ORF-1p promoted *Bcl-2* gene expression which is involved in resistance to apoptosis, and inhibited the expression of the genes, *p15*, *p27* and *p53*<sup>[13]</sup>. These genes promote apoptosis and arrest cell growth or cell cycling. These results were consistent with those from the luciferase assays. LINE-1 ORF-1p reduced the activity of Luc-p21, Luc-SBE, and Luc-Sp1 reporters and enhanced Luc-ARE reporter transcription. p53, Smad4, Sp1 and AR are important transcription factors, which modulate the transcription of many genes and play a role in the regulation of cancer progression. LINE-1 ORF-1p may interact with these transcriptional factors which are recruited to specific regulatory elements such as SP1 and p53 binding sites. They then interfere with their function through conformational alteration or by affecting their co-repressor recruitment. Further studies are required to address this hypothesis.

Paclitaxel targets microtubules. Overexpression of LINE-1 ORF-1p did not antagonize the cytotoxic effect of paclitaxel, however, knockdown of LINE-1 ORF-1p and paclitaxel had a synergistic effect and reduced its expression level and slowed cell growth by modulating *p53*, *p15*, *p27*, and *Bcl-2* expression.

Taken together, these findings suggest that LINE-1 ORF-1p may have a role in the development of HCC, and is likely to be involved in the prediction of chemotherapeutic effect. In addition, these findings significantly advance our understanding of the functions and mechanisms of LINE-1 ORF-1p in cancer cell proliferation and may provide a novel potential therapeutic target in HCC.

## COMMENTS

### Background

Hepatocellular carcinoma (HCC) is the commonest form of cancer worldwide, and the third most common cause of cancer death. Chemotherapy is currently the most commonly adopted strategy for treating HCC patients; however, it also has shortcomings as HCC patients can exhibit robust multidrug resistance.

### Research frontiers

Long interspersed nuclear element-1 ORF-1 protein (LINE-1 ORF-1p) plays an important role in the development of several types of carcinoma. However, the involvement of LINE-1 ORF-1p in the pathogenesis of hepatocellular carcinoma and whether it is responsible for making these tumors resistant to chemotherapy has not been addressed. In this study, the authors demonstrate that the overexpression of LINE-1 ORF-1p, commonly observed in HCC patients, could be a potential mechanism for both promoting cell proliferation and for resistance to chemotherapy.

### Innovations and breakthroughs

Recent reports have highlighted the importance of LINE-1 ORF-1p in different types of cancers. This is the first study to report that LINE-1 ORF-1p mediates resistance to chemotherapy in HCC patients. Furthermore, our *in vitro* studies suggest that LINE-1 ORF-1p mediates the regulation of cellular apoptotic and proliferative pathways, and aids in the metastatic progression of HCC.

### Applications

By understanding the mechanism of LINE-1 ORF-1p-mediated induction of resistance to chemotherapy, this may represent a future strategy for therapeutic intervention in the treatment of patients with hepatocellular carcinoma. In addition, the expression levels of LINE-1 ORF-1p in hepatocellular carcinoma patients may serve as a predictive marker of chemotherapy outcome.

### Terminology

LINE-1 is an autonomous non-LTR retrotransposon in mammals. Retrotransposition requires the function of the two L1-encoded polypeptides, ORF1p and ORF2p. LINE-1 ORF-1p, which comprises at least 17% of the human genome sequence, is silenced (non-active) in normal adult tissues. However, in cancer cells, LINE-1 ORF-1p is active and affects genomic stability and chromosome structure through its retrotransposon activity, eventually leading to tumorigenesis.

### Peer review

In this work, the authors investigated the roles of LINE-1 ORF-1p in the drug resistance and proliferation of HCC with interesting findings.

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## Papillary thyroid cancer and inflammatory bowel disease: Is there a relationship?

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### Abstract

**AIM:** To formally study age of diagnosis of papillary thyroid cancer (PTC) in inflammatory bowel disease (IBD) patients and evaluate the prevalence of PTC in IBD patients compared to a control population.

**METHODS:** We were interested in testing the hy-

pothesis that patients with IBD are more likely to be diagnosed with PTC than a control population. A retrospective cohort analysis was performed using the University of Pennsylvania Health System's electronic database. Outpatients from 1998-2009 were included in the search, and patients in the cohort were selected based on ICD-9 codes. Inclusion criteria included the diagnosis of Crohn's disease (CD) or ulcerative colitis (UC) and the concurrent diagnosis of thyroid cancer in comparison to a control population. Using these methods 912 patients with CD and 1774 with UC were compared to 1638 diverticulitis and 19 447 asthma controls. Statistics were performed using corrected chi-square analysis. The primary outcome for this study was the diagnosis of PTC. Approval to conduct this study was obtained by the Institutional Review Board at the University of Pennsylvania.

**RESULTS:** The mean age was 47.5 years (range: 18-102 years) and 66% patients were female. An analysis of variance model was used to compare the age of PTC diagnosis between the CD, UC, asthma and diverticulitis groups, and a statistically significant difference in age at PTC diagnosis was noted across all groups ( $F = 6.35$ ,  $df = 3$ ,  $P = 0.0006$ ). The age of PTC diagnosis in CD patients was statistically significantly lower than UC, asthma, and diverticulitis patients (average PTC diagnosis age for CD 25, UC 49, asthma 45, diverticulitis 63). After covarying for sex and age in 2009, the difference in age at PTC diagnosis remained statistically significant ( $F = 4.13$ ,  $df = 3$ ,  $P = 0.0089$ ). A total of 86 patients were diagnosed with PTC. Nine patients (0.5%) with UC were diagnosed with PTC. Patients with UC were not shown to be more likely to develop PTC [odds ratio (OR): 1.544, 95%CI 0.767-3.108] compared to asthma controls. Four patients (0.4%) with CD were diagnosed with PTC. Patients with CD were not shown to be more likely to develop PTC (OR: 1.334, 95%CI 0.485-3.672) compared to a control population with asthma. Nine patients (0.5%) with a history of diverticulitis were diagnosed with PTC. Pa-

tients with diverticulitis were not shown to be more likely to develop PTC (OR: 1.673, 95%CI 0.831-3.368) compared to asthma controls. Patients with CD or UC were not less likely to develop PTC compared to those with diverticulitis (CD OR: 0.80, 95%CI 0.25-2.60; UC OR: 0.92, 95%CI 0.37-2.33). None of the patients used immunosuppressant medications prior to the diagnosis of PTC (azathioprine, 6-mercaptopurine, and methotrexate).

**CONCLUSION:** There is a significant difference in age of diagnosis of PTC in patients with CD compared to patients with UC and the control populations studied.

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**Key words:** Papillary thyroid cancer; Inflammatory bowel disease; Crohn disease; Ulcerative colitis

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## INTRODUCTION

Inflammatory bowel diseases (IBD), specifically Crohn's disease (CD) and ulcerative colitis (UC), are idiopathic chronic inflammatory disorders resulting from defects in the barrier function of the intestinal epithelium and inappropriate activation of the mucosal immune system. IBD is also associated with several other extra-intestinal disease states, including ankylosing spondylitis, arthritis, pyoderma gangrenosum, and uveitis<sup>[1]</sup>. Less is known about the association of IBD with diseases of the thyroid.

Early studies have suggested a relationship between thyroid abnormalities and IBD<sup>[2]</sup>. For example, CD has been associated with Graves' disease and Hashimoto's thyroiditis<sup>[3]</sup>. However, the association with IBD and thyroid cancer has been less critically analyzed. Thyroid cancer is the most common endocrine cancer, with over 80% of all cases consisting of the papillary type. It is more common in women than in men (3:1 ratio) and is more common in whites than in African-Americans. The mean age of diagnosis is 40 years old<sup>[4]</sup>. There are several case reports of thyroid cancer in patients with IBD, two of them involving UC<sup>[5,6]</sup>. Moss *et al*<sup>[7]</sup> described five cases of papillary thyroid cancer (PTC) in patients with preexisting CD. Despite these anecdotal published findings, there have been no formal studies evaluating the relationship that exists between PTC and IBD in a population cohort study.

It is uncertain whether PTC in patients with IBD is due to an association or coincidence. Knowledge of a relationship is important in order to determine the factors

that link IBD and PTC. Exposure to radiation and multi-vitamin intake are both considered risk factors for PTC, both of which are common in patients with IBD due to diagnostic studies and malnutrition respectively<sup>[8]</sup>. However, a recent study by Peloquin *et al*<sup>[9]</sup> concluded that the radiation exposure in an IBD population was equivalent to the average annual background radiation dose from naturally occurring sources in the United States, while a smaller subset of patients had substantially higher doses. In addition, immunosuppressants such as azathioprine and 6-mercaptopurine are commonly taken by patients with IBD to induce remission and can increase the risk of malignancy, specifically lymphoproliferative disorders<sup>[10,11]</sup>. Whether these medications increase the risk of thyroid cancer is unknown.

It is already established that an inappropriate inflammatory response results in IBD. Defects in genes involving the innate immune system and activation of inflammatory cytokines such as NOD2, autophagy-related 16-like 1, and interleukin-23 lead to intestinal inflammation<sup>[12,13]</sup>. Similarly, lymphocytic infiltration is seen in PTC, suggesting that immunologic factors may contribute to neoplastic development<sup>[14]</sup>. Thus, the inflammatory modulators involved in the pathogenesis of IBD may have an effect on PTC development.

This study addressed an important question regarding the epidemiology of IBD and PTC. The specific aim of this study was to identify any relationships between the two diseases and to determine whether patients with IBD have a higher prevalence of PTC than a control population. Knowledge of an association if present has potential to impact the management of patients with IBD, which may warrant screening for thyroid cancer through regular thyroid examinations.

## MATERIALS AND METHODS

### Definition of the cohort

A retrospective cohort study from within the University of Pennsylvania Health System was performed using the data from the electronic health system database (EPIC Hyperspace Summer 2009 IU 6, EPIC Systems Corporation, Verona, Wisconsin, United States). Patients who were seen as outpatients in our health system from 1998 to 2009 were included in the search. Patients with CD and UC were identified based on ICD-9 codes (ICD-9 codes for CD: 555, 550.0, 555.1, 555.9; codes for UC: 556, 556.0, 556.1, 556.2, 556.3, 556.5, 556.6, 556.8, 556.9).

Inclusion criteria included the diagnosis of CD or UC and the concurrent diagnosis of thyroid cancer. The latter diagnosis was identified using the ICD-9 code for thyroid cancer: 193. The papillary type of thyroid cancer was verified using biopsy reports or the patients' medical records. Patients who met inclusion criteria were included in the final analysis and were compared to patients with inflammatory controls with diverticulitis and asthma. Controls were identified based on a search of our electronic database using ICD-9 codes (ICD-9 code

**Table 1** Concomitant diagnosis of papillary thyroid cancer

Diagnosis	No. of patients diagnosed with PTC	Unadjusted OR (95%CI) compared to asthma controls	OR (95%CI) compared to asthma controls, adjusted for sex and age in 2009
CD	4	1.334 (0.485-3.672)	1.510 (0.548-4.162)
UC	9	1.544 (0.767-3.108)	1.679 (0.832-3.389)
Diverticulitis	9	1.673 (0.831-3.368)	1.338 (0.644-2.779)
Asthma	64	NA	NA

OR: Odds ratio; PTC: Papillary thyroid cancer; NA: Not available.

for diverticulitis: 562.11, 562.13; code for asthma: 493). Approval was obtained from the Institutional Review Board at the University of Pennsylvania to perform our study.

### Definition of outcome

The primary outcome for this study was a diagnosis of PTC. We did not limit our outcome definition to an incident diagnosis of PTC following a diagnosis of IBD. We were interested in testing the hypothesis that patients with IBD are more likely to be diagnosed with PTC than a control population, not that IBD in and of itself predisposes patients to PTC. As such, we searched for any diagnosis of PTC at any time in the patients' records.

### Statistical analysis

All analyses were performed separately for patients with CD and UC, and then as a combined IBD group. Analyses began with descriptive statistics. Continuous variables were reported as means  $\pm$  SD. Categorical variables were reported as counts and percentages. Since age was approximately normally distributed, we used analysis of variance to test for differences in the mean age at diagnosis of PTC<sup>[13]</sup>. Statistical analysis was performed at the University of Pennsylvania, Philadelphia, PA using SAS software version 9.2 (SAS Institute, Cary, NC).

To estimate the association between PTC and IBD in this cross-sectional study, we calculated the odds ratio (OR) and 95%CI using logistic regression. We considered a finding to be statistically significance when  $P < 0.05$ .

### Sample size and power calculations

A two group continuity corrected  $\chi^2$  test with a 0.050 two-sided significance level had 80% power to detect the difference between a proportion of 0.3% in the asthma group and a proportion of 0.8% in the UC group (OR of 2.41) when the sample sizes are 19 447 and 1774, respectively (a total sample size of 21 221). A two group continuity corrected  $\chi^2$  test with a 0.050 two-sided significance level had 80% power to detect the difference between a proportion of 0.3% in the asthma group and a proportion of 1.0% in the UC group (OR of 3.02) when the sample sizes are 19 447 and 912, respectively (a total sample size of 20 359).

## RESULTS

Our retrospective analysis yielded a total of 2686 patients who were diagnosed with either CD or UC. A total of 912 patients with CD and 1774 patients with UC met inclusion criteria and were included in our analysis. These patients were compared to 1638 diverticulitis and 19447 asthma controls.

The overall mean age was 47.5 years (range: 18-102 years) and 66% patients were female. Using an analysis of variance model to compare the age of thyroid diagnosis between the CD, UC, asthma and diverticulitis groups, a statistically significant difference in age at PTC diagnosis was noted across all groups ( $F = 6.35$ ,  $df = 3$ ,  $P = 0.0006$ ). Of note, the age of PTC diagnosis in CD patients was statistically significantly lower than UC, asthma, and diverticulitis patients (average PTC diagnosis age for CD: 25 years, UC 49 years, asthma: 45 years, diverticulitis: 63 years). After covarying for sex and age in 2009, the difference in age at PTC diagnosis remained statistically significant ( $F = 4.13$ ,  $df = 3$ ,  $P = 0.0089$ ).

A total of 86 patients were diagnosed with PTC. Nine patients (0.5%) with UC were diagnosed with PTC. Patients with UC were not shown to be more likely to develop PTC (OR: 1.544, 95%CI 0.767-3.108) compared to asthma controls. Four patients (0.4%) with CD were diagnosed with PTC. Patients with CD were not shown to be more likely to develop PTC (OR: 1.334, 95%CI 0.485-3.672) compared to a control population with asthma. Nine patients (0.5%) with a history of diverticulitis were diagnosed with PTC. Patients with diverticulitis were not shown to be more likely to develop PTC (OR: 1.673, 95%CI 0.831-3.368) compared to asthma controls (Table 1).

Patients with CD or UC were not less likely to develop PTC compared to those with diverticulitis (CD OR: 0.80, 95%CI 0.25-2.60; UC OR: 0.92, 95%CI 0.37-2.33). None of the patients used immunosuppressant medications prior to the diagnosis of PTC (azathioprine, 6-mercaptopurine, and methotrexate).

## DISCUSSION

The results of our present study highlights a statistically significant difference in age of diagnosis of PTC in patients with CD compared to patients with UC and the control populations studied. Patients with CD are diagnosed with PTC at a much younger age, possibly due to a unique pathogenetic mechanism of disease occurring in this subset of patients. It is established that CD results from a variety of factors, including genetic, environmental and immunological factors. An inappropriate inflammatory reaction is a key factor in the pathogenesis of CD. Similarly, PTC involves an inflammatory reaction, in particular, a lymphocytic infiltration of thyroid tissue<sup>[14]</sup>. Thus, overlapping immunologic factors/pathways may connect these two diseases together. The inflammatory modulators involved in the pathogenesis of IBD



may have an effect on PTC development or PTC may predispose patients to developing IBD.

In addition there have been specific genetic mutations described to be present in patients with CD. The initial frameshift mutation described was in the *NOD-2* gene in 2001<sup>[16]</sup>. Data from epidemiological studies, based on concordance data in family studies via linkage analysis to genome-wide association studies, highlight evidence for over 30 distinct genomic loci involved in the genetic susceptibility to CD. These loci encode genes involved in a number of homeostatic mechanisms: innate pattern recognition receptors, the differentiation of Th17-lymphocytes, autophagy, maintenance of epithelial barrier integrity, and the orchestration of the secondary immune response<sup>[17]</sup>. It is perceived that recognition of these loci will help to improve our understanding of the pathophysiology of CD.

Similarly, there have been several genetic mutations described in patients with PTC, the most common involving the RET/papillary thyroid carcinomas 1 (PTC1) and RET/PTC3 rearrangements which account for more than 90% of all genetic rearrangements found in PTC, and have been found in 30%-40% of adult patients with sporadic PTC in the United States, Italy, and Canada<sup>[18]</sup>. Other genetic mutations include the neurotrophic tyrosine kinase, receptor, type 1 (NTRK1) rearrangement, Ras kinases and Raf kinases<sup>[19-23]</sup>. In addition, recent literature has incited the presence of 7 gene regions to be associated with PTC<sup>[24]</sup>. These results suggest a possible role of genes involved in maintenance of genomic integrity in relation to risk of PTC.

Several studies determined significantly higher oncogenic rearrangements of RET and NTRK1 proto-oncogenes in patients with PTC that occurred primarily in children and young adults. An analysis of 92 patients with PTC determined that patients age 4-30 years had significantly higher frequency of RET or NTRK1 gene compared to those 31-80 years (57% *vs* 32%,  $P = 0.019$ ). Further, among patients ages 4-19 years, 67% of them displayed RET gene rearrangement<sup>[25]</sup>. Studies by Jhiang *et al.*<sup>[26]</sup> and Soares *et al.*<sup>[27]</sup> found that patients displaying RET rearrangement in their PTC had significantly lower mean age at diagnosis than those whose PTC did not present this rearrangement (32 years *vs* 50 years,  $P < 0.05$  and 28 years *vs* 45 years,  $P = 0.005$ , respectively)<sup>[26,27]</sup>. An assessment of spontaneous PTC in 33 young patients ages 6-21 years showed that the RET/PTC1 rearrangement was common in the sporadic form of children with PTC (53%), with the RET/PTC3 mutation more commonly seen among children with PTC secondary to radiation exposure (67%-76%) as reported in prior studies<sup>[28-31]</sup>.

One limitation to this study is the small number of patients who were found to have PTC. With so few patients with both diagnoses, it was difficult to elucidate a temporal relationship between the two diseases. Some patients were diagnosed with PTC followed by a CD diagnosis, and vice versa. A larger study would help establish whether there is a diagnosis pattern. The find-

ings on prevalence do not suggest a higher prevalence of PTC in patients with IBD compared to the control populations studied. We cannot exclude the potential for a type II error given that our study was underpowered. A study using a much larger cohort of patients would be better-powered and would more likely lead to statistically significant results and better define a lack of relationship between the two diseases.

There were several other limitations to this study. We did not test for confounding; we did not examine other thyroid conditions including Graves disease, Hashimoto's thyroiditis, goiter, and non-papillary type thyroid cancer as potential confounder variables. The other limitation of our study is that some relevant clinical information from the past medical history of patients may not have been included in the electronic database and thus may have been potentially omitted. This is a common limitation of retrospective studies.

In summary, the results of our study suggest that patients with CD who are also diagnosed with PTC are diagnosed at a much younger age than patients with UC and other controls. This is important for cancer detection in CD patients. Currently age, gender, radiation exposure, and a low iodine diet are the only known clinical risk factors for PTC; further studies are needed to establish whether patients with CD are at increased risk of developing PTC. If there is an increased risk, then more stringent methods of cancer screening may be warranted for CD patients, including initiating screening at a younger age.

## COMMENTS

### Background

Inflammatory bowel diseases (IBD), specifically Crohn's disease (CD) and ulcerative colitis (UC), are idiopathic chronic inflammatory disorders resulting from defects in the barrier function of the intestinal epithelium and inappropriate activation of the mucosal immune system. Early studies have suggested a relationship between thyroid abnormalities and IBD. For example, CD has been associated with Graves' disease and Hashimoto's thyroiditis. However, the association with IBD and thyroid cancer has been less critically analyzed. The aim of this study was to formerly study the age of diagnosis of papillary thyroid cancer (PTC) in IBD patients and evaluate the prevalence of PTC in IBD patients compared to a control population.

### Research frontiers

This study addressed an important question regarding the epidemiology of IBD and PTC. The results suggest that CD patients are diagnosed with PTC at a much younger age, possibly due to a unique pathogenetic mechanism of disease occurs in this subset of patients.

### Innovations and breakthroughs

The researchers drew a conclusion that there is a significant difference in age of diagnosis of PTC in patients with CD compared to patients with UC and the control populations studied. Patients with CD are diagnosed with PTC at a much younger age, possibly due to a unique pathogenetic mechanism of disease occurs in this subset of patients. It is established that CD results from a variety of factors, including genetic, environmental and immunological factors. An inappropriate inflammatory reaction is a key factor in the pathogenesis of CD. Similarly, PTC involves an inflammatory reaction, in particular, a lymphocytic infiltration of thyroid tissue. Thus, overlapping immunologic factors/pathways may connect these two diseases together. The inflammatory modulators involved in the pathogenesis of IBD may have an effect on PTC development or PTC may predispose patients to developing IBD.

## Applications

This study answers important questions regarding the epidemiology of PTC and IBD, and also addresses the topic of cancer detection in CD patients. Currently age, gender, radiation exposure, and a low iodine diet are the only known clinical risk factors for PTC; further studies are needed to establish whether patients with CD are at increased risk of developing PTC. If there is an increased risk, then more stringent methods of cancer screening may be warranted for CD patients, including initiating screening at a younger age.

## Peer review

The paper evaluated on a retrospective cohort study prevalence of PTC in IBD patients compared to a control population. This is a very interesting subject and there are many lacunae in the literature.

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## Efficacy and safety of Chlorella supplementation in adults with chronic hepatitis C virus infection

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### Abstract

**AIM:** To evaluate the safety and efficacy of Chlorella in 18 patients chronically infected with hepatitis C virus (HCV) genotype 1.

**METHODS:** Eighteen adults with chronic infection by HCV genotype 1 received daily oral supplementation of Chlorella for 12 wk. Changes in the RNA levels of HCV, as well as those of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were evaluated following this treatment period. Paired *t* tests were conducted to compare the means of the different variables at the beginning and end of the study. Side effects and quality of life aspects were also compared between weeks 0 and 12 of the study period.

**RESULTS:** A majority 84.61% of the patients had a significant decrease in their ALT levels from week 0 to week 12. Evaluation of side effects showed that Chlorella was well tolerated. Quality of life assessment showed that 76.9 of the participants reported an improvement in their energy levels and 46.1% reported an improvement in their perception of general health. Although 69.23% also showed a decrease in their AST

levels, this was not statistically significant. Most patients that exhibited an improvement in their ALT and AST levels also showed a tendency toward a decreased HCV viral load. The HCV RNA levels showed a decrease in 69.23% of the patients, which along with changes in AST/ALT ratios from week 0 to week 12, these results were not statistically significant.

**CONCLUSION:** Chlorella supplementation was well tolerated in patients with chronic HCV and associated with a significant decrease in ALT liver enzyme levels.

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**Key words:** Chlorella; Hepatitis C virus; Interferon; Aspartate and alanine aminotransferase; Ratio

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### INTRODUCTION

Complementary and alternative medicines to treat chronic liver diseases, including chronic hepatitis C virus (HCV) infection, are becoming increasingly popular in North America<sup>[1-5]</sup>. Infection with HCV is global in nature, infecting approximately 160 million persons worldwide or roughly 2% of the world population, with some countries documenting a rate of 15% or more<sup>[6,7]</sup>. After an initial HCV infection, close to 70% of cases develop chronic infection that may progress to liver cirrhosis and hepatocellular carcinoma if left untreated<sup>[8]</sup>. The successful treatment of chronic HCV infection is determined by a reduced HCV-RNA viral load and improved liver function and histology. The current Food and Drug



Administration approved treatment for HCV is up to 42 wk of interferon and antiviral medications. However, there are significant costs associated with these medications and side effects which limit their use thus stressing the need for novel treatment options<sup>[9,10]</sup>. In addition, subjects that fail to respond to the initial treatment are less likely to respond to retreatment<sup>[11]</sup>. Many herbal and other natural compounds have now been used for the treatment of liver diseases, including HCV infection. Silybum marianum and Lactoferrin have been associated with a decrease in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels<sup>[12,13]</sup>. More recently, green tea catechins have been shown to inhibit HCV attachment and transmission in human liver cells *in vitro*<sup>[14]</sup>. However, the potential benefits of herbal and other natural molecules in inhibiting the progression of HCV infection are only beginning to be understood, and more controlled studies are needed in this area.

Chlorella, a fresh water unicellular alga rich in macro- and micronutrients, has been used as a food source and nutritional supplement for centuries<sup>[15]</sup>. In animals, Chlorella has been reported to improve host resistance to viral infection and tumors<sup>[16-18]</sup>. In humans, Chlorella supplementation has been shown to enhance the antibody titer after influenza immunization<sup>[19]</sup>, and to improve the outcome in several chronic diseases<sup>[20,21]</sup>. Chlorella supplementation has been also associated with an improvement in liver function in animal models<sup>[22,23]</sup>. Due to the documented benefits of Chlorella treatments in liver diseases in animals and in chronic diseases in humans, we studied the effects of Chlorella in 18 patients with chronic HCV genotype 1 infection. The plasma HCV RNA levels, hematological and chemistry results, including liver enzyme levels, and the quality of life and psychological well-being were assessed in this cohort following dietary supplementations with Chlorella-derived products.

## MATERIALS AND METHODS

### Population

The current study trial took place at a primary care clinic in western Massachusetts. Approval was obtained from the New England Institutional Review Board (NEIRB, Wellesley, MA, United States). The study cohort comprised 18 patients with chronic HCV infection who were either unwilling or unable to receive an interferon plus antiviral therapy. The inclusion criteria were an age of 18-65 years, evidence of chronic HCV infection by reverse transcription polymerase chain reaction (PCR), and a confirmation that the infection was due to HCV genotype 1. The exclusion criteria were any acute or chronic liver disease other than chronic HCV infection, any evidence of advanced liver disease such as a history or presence of ascites, a history of bleeding esophageal varices or encephalopathy, any known existing medical condition that could interfere with participation in the current

study, co-infection with hepatitis B virus and/or human immunodeficiency virus, or a history of active alcohol or drug abuse three months prior to the beginning of the trial. Some of the patients had already been treated with interferon plus ribavirin (three years or more previously) before their participation in this study and had failed to respond.

### Study design

This study examined the effects of Chlorella upon the HCV viral load, and on hematological and chemical test results, including AST and ALT liver enzyme levels, in infected patients during a 12 wk treatment period. To control for factors others than Chlorella that could have affected the results of our study, only patients with a HCV genotype 1 infection were selected for this trial. The AST/ALT ratio at week 0 and after the 12 wk of Chlorella supplementation was compared among the 13 subjects in the cohort.

We also compared the safety and efficacy of orally administered Chlorella by assessing the presence of possible side effects and their impact on quality of life. The side effects assessed included constipation, diarrhea, depression, irritability, headache/body aches, and any other significant symptoms that arose during the study period. Similarly, quality of life was assessed by evaluating changes in energy levels, general health perceptions, quality of sleep and changes in appetite. Patients were interviewed about possible side effects and impact of quality of life at baseline, and at weeks 1, 2, 4, 8 and 12. The answers were coded on a 5 level scale from 1 (much worse) to 5 (much better). Scores were then compared between baseline and weeks 1, 2, 4, 8 and 12. To assess compliance, patients were instructed to return all used and unused products at each visit.

“Sun Chlorella A™” consisted of a dry pulverized Chlorella pyrenoidosa powder plus a water soluble extract (“Wakasa Gold™”; Sun Chlorella Corp, Kyoto, Japan), which contained 82 mg/mL of Chlorella. It was administered orally to each patient as follows: three 500 mg tablets were administered twice daily on days 1-7 and then three times each day thereafter. Wakasa Gold™ was administered at a dose of 30 mL twice a day starting on day 1. Both pulverized tablets and water soluble extracts were used in the treatment given in order to administer the greatest amount of Chlorella possible. Patients were evaluated as described above during the duration of the trial. Routine hematology, chemical tests and HCV RNA levels were done at baseline and at week 12 using a PCR-based assay conducted at a local laboratory (Life Lab; Mercy Hospital, Springfield, MA, United States).

Changes in AST and ALT liver enzyme levels and the HCV viral load in our “Chlorella treat” cohort were measured after the 12 wk study period and were compared with the same laboratory test results in a control group of 26 subjects who were also chronically infected with HCV genotype 1, but did not receive Chlorella. Subjects

**Table 1** Levels of aspartate aminotransferase, alanine aminotransferase, aspartate/alanine aminotransferase ratio and hepatitis C virus viral load at weeks 0 and 12

Patient No.	AST (IU/mL)		ALT (IU/mL)		AST/ALT ratio		HCV viral load (IU/mL)		Previous INF Rx
	Week 0	Week 12	Week 0	Week 12	Week 0	Week 12	Week 0	Week 12	
1	80	63	82	68	0.975	0.926	11 420	1183	No
2	53	44	103	72	0.514	0.611	140 642	32 334	No
3	87	40	71	39	1.225	1.025	91 808	24 141	No
4	35	56	48	92	0.729	0.608	223 075	89 331	No
5	19	25	22	21	0.863	1.19	221 116	281 886	No
6	39	33	83	72	0.469	0.454	1 917 040	560 082	No
7	51	41	81	64	0.629	0.645	269 079	217 334	Yes
8	138	74	157	105	0.878	0.704	382 773	1 302 860	Yes
9	48	51	37	47	1.297	1.085	136 292	236 748	Yes
10	75	85	102	95	0.735	0.894	716 152	503 764	Yes
11	27	24	39	29	0.692	0.827	7 672 080	7 692 310	Yes
12	103	77	157	119	0.656	0.647	847 412	542 398	Yes
13	127	93	159	106	0.798	0.877	869 846	568 398	Yes

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; HCV: Hepatitis C virus; INF: Interferon.

in the control group were randomly selected, attended the same clinic and had a similar demographic profile as the Chlorella-treated group. Changes in the hematological, liver enzyme values and HCV-RNA viral load in this control group were assessed within an 11-21 wk period as part of a standard clinical evaluation. No subjects in either the study or control groups received treatment with interferon or other anti-viral drugs during the study period. This study was conducted in accordance with the Declaration of Helsinki, Good Clinical Practices and the study protocol was reviewed and approved by the NEIRB.

### Statistical analysis

Data gathered during the study was used to examine statistically significant changes in the HCV RNA levels, and in the hematological and liver enzyme (AST, ALT) levels. Side effects and quality of life aspects were also compared between weeks 0 and 12 of the study period. Paired *t* tests were conducted to compare the means of the different variables at the beginning and end of the study. *P* values < 0.05 were considered statistically significant. All data was analyzed using Statistical Package for Social Sciences version 17.0.

## RESULTS

Thirteen out of 18 enrolled patients completed our 12 wk study. Of the five patients who did not complete the trial, one individual discontinued treatment due to constipation on the first two days of treatment, which resolved upon treatment withdrawal. Four subjects were excluded due to poor compliance which was unrelated to side effects or changes in their general health status.

### HCV RNA titer and liver enzyme analysis

Changes in the HCV RNA levels and in the AST and ALT liver enzyme levels among our study subjects are shown in Table 1. A majority of patients experienced

an improvement in their liver enzyme profiles; 84.61% (11/13) had a decrease in their ALT levels from week 0 (mean  $\pm$  SD, 89.30%  $\pm$  49.36%) to week 12 (mean  $\pm$  SD, 71.46%  $\pm$  31.12%). Moreover, this decrease was statistically significant (*P* < 0.05). Although 69.23% of the patients (9/13) also showed a decrease in their AST levels from week 0 (mean  $\pm$  SD, 67.84%  $\pm$  37.73%) to week 12 (mean  $\pm$  SD, 54.30%  $\pm$  22.63%), this was not statistically significant (*P* = 0.06). The results further showed that 69.23% (9/13) of the patients had a decrease in their HCV RNA levels, although the results of the paired *t* test also showed this was not statistically significant (*P* = 0.42). Most patients that exhibited an improvement in their AST and ALT levels also showed a tendency toward a decreased HCV viral load. An exception was patient No. 8 whose viral load increased significantly (Table 1). The changes in the AST/ALT ratio between weeks 0 and 12 among the subjects in the Chlorella cohort shown in Table 1 were not statistically significant. The values obtained from additional laboratory tests, including routine hematology (complete blood count and differential) and chemical tests, showed no statistically significant changes during the study period (data not shown). No statistically significant differences in the changes of the AST and ALT liver enzymes, AST/ALT ratio and HCV viral load was observed within a 11-21 wk period, among the control group who received not Chlorella supplementation.

### Side effect profile and quality of life assessment

The main side effects associated with the Chlorella treatments in our trial included constipation and diarrhea. Four of the 13 patients (30.7%) with a previous history of constipation reported their symptoms as worse or somewhat worse during the first two weeks of treatment. However, in all of these cases, the constipation symptoms were mild to moderate and resolved within the first two weeks. These patients continued in the trial for the scheduled 12 wk. Similarly, two of the 13 patients (15.3%) who completed the study complained of mild

diarrhea at week 1, but reported that these symptoms were much improved after week 2. None of the patients reported symptoms of abdominal pain, fever, depression, headache, body ache or other complaints during the 12 wk study.

### Quality of life

Four variables were tested to assess changes in quality of life; 76.9 % of the patients (10/13) reported an improvement in their energy levels during the study period whereas 23.1% (3/13) reported no change in energy levels. In addition, 46.1% (6/13) of the patients reported an improvement in their perception of general health while 53.9% (7/13) reported no change in this regard. None of the patients reported issues with sleep quality or appetite during the 12 wk study period.

## DISCUSSION

In our present study, most of the subject patients with chronic HCV showed a good tolerance to Chlorella oral supplementation. This was anticipated, based on previous studies which had also shown good tolerance of similar doses of Chlorella administered in pregnant, lactating<sup>[24]</sup>, and elderly subjects<sup>[19]</sup>, as well as in patients with diverse chronic diseases<sup>[20,21]</sup>. In addition, a significant percentage of our subjects reported health improvements in their quality of life questionnaire: 76.9% had an increase in energy levels and 46.1 % described an improvement in their general health perception.

The most significant finding from our current study was the statistically significant decrease in ALT levels, a marker of liver inflammation, among our patient cohort after 12 wk of Chlorella treatment. The cause-effect relationship between the observed significant decrease in the ALT levels and treatment with Chlorella is further suggested by the lack of significant ALT changes within the 11-21 wk period in a control group of HCV genotype 1 infected patients who had not received Chlorella. We further found that 69.23% of our patients showed a tendency toward a decrease in both their AST and HCV RNA levels after the 12 wk Chlorella treatment period, although this was not statistically significant. The AST/ALT ratio has been widely used as an indicator of liver disease; a ratio higher of 1 is used as indicator of liver fibrosis and cirrhosis<sup>[25,26]</sup>. In our cohort only four of thirteen subjects had an AST/ALT higher than 1 and there was no significant change after the Chlorella supplementation period (Table 1).

Animal studies have shown that enhanced immunocompetence provided by oral administration of Chlorella extract is mediated by augmentation of cell mediated immunity in normal and immunocompromised hosts<sup>[16-18]</sup>. More recently Kwak *et al*<sup>[27]</sup> demonstrated enhancement of natural killer cell activity and increase interferon and other cytokines production in humans after an 8 wk period of Chlorella supplementation at doses similar

to the ones used in this study. We propose that the improvements in liver function tests in our population with chronic HCV infection is most likely due to the beneficial immunostimulatory effect of Chlorella supplementation. This is consistent with the finding that in our cohort most of the subjects that had been previously treated with interferon and failed to respond, were less likely to show a decrease in ALT and HCV-RNA values during Chlorella supplementation (Previous INF Rx, Table 1). The recovery from liver inflammation in all viral and non-viral cases of hepatitis is associated with a reduction in the ALT levels<sup>[28-30]</sup>. In Chronic HCV infection, early normalization of the ALT levels is predictive of the response to interferon<sup>[9,10,30]</sup>. However, whether the significant decrease in ALT values observed in our patients after 12 wk of Chlorella administration is associated with an improvement in liver histology remains to be determined.

The strength of our hypothesis; that the benefits of Chlorella supplementation are related to the well known immunoenhancement effects of Chlorella is limited by the lower number of subjects in our cohort and the limited time of supplementation (12 wk). Nevertheless, we conclude from our present findings that the benefits of Chlorella administration in the treatment of chronic HCV infection before and/or during the administration of interferon plus antiviral drugs, as well as the effects of Chlorella upon chronic infection by other HCV genotypes, warrant further study.

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## COMMENTS

### Background

Chronic hepatitis C viral (HCV) infection are becoming increasingly popular in North America and most cases develop chronic infection that may progress to liver cirrhosis and hepatocellular carcinoma if left untreated. Chlorella supplementation has been shown to enhance the antibody titer after influenza immunization, and to improve the outcome in several chronic diseases.

### Research frontiers

Animal studies have shown that enhanced immunocompetence provided by oral administration of Chlorella extract is mediated by augmentation of cell mediated immunity in normal and immunocompromised host. More recently Kwak *et al* demonstrated enhancement of natural killer cell activity and increase interferon and other cytokines production in humans after an 8 wk period of Chlorella supplementation at doses similar to the ones used in this study.

### Innovations and breakthroughs

The study suggests that the benefits of Chlorella administration in the treatment of chronic HCV infection before and/or during the administration of interferon plus antiviral drugs, as well as the effects of Chlorella upon chronic infection by other HCV genotypes, warrant further study.

### Terminology

Chlorella, a fresh water unicellular alga rich in macro- and micronutrients, has been used as a food source and nutritional supplement for centuries. In animals,



Chlorella has been reported to improve host resistance to viral infection and tumors.

### Peer review

The results are interesting and suggest that most of the subject patients with chronic HCV showed a good tolerance to Chlorella oral supplementation.

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## Efficacy of infliximab in acute severe ulcerative colitis: A single-centre experience

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### Abstract

**AIM:** To suggest infliximab (IFX) is effective for acute severe ulcerative colitis, from real-life clinical practice.

**METHODS:** All patients receiving IFX for the treatment of acute severe ulcerative colitis in a single centre were included. Data were extracted from clinical records in order to assess response to IFX therapy. The primary endpoint was colectomy-free survival, and secondary outcomes included glucocorticosteroid-free remission and safety, which was evaluated by recording deaths and adverse events. Demographic and clinical characteristics of those who underwent colectomy and those who were colectomy-free, both at discharge from their index admission, and during follow-up after an initial response to IFX were compared.

**RESULTS:** Forty-four patients (16 females, mean age 36 years) received IFX between May 2006 and January

2012 for acute severe ulcerative colitis. The median duration of follow-up post-first infusion was 396 d (interquartile range = 173-828 d). There were 21 (47.7%) patients with < 1 year of follow-up, 10 (22.7%) with 1 years to 2 years of follow-up, and 13 (29.5%) with > 2 years of follow-up post-first infusion of IFX. Overall, 35 (79.5%) responded to IFX, avoiding colectomy during their index admission, 29 (65.9%) were colectomy-free at last point of follow-up (median follow-up 396 d), and 25 (56.8%) were in glucocorticosteroid-free remission at end of follow-up. There was one death from post-operative sepsis, 20 d after a single IFX infusion. Colectomy rates were generally lower among those "bridging" to thiopurine. Of 18 patients "bridged" to thiopurine therapy, 17 (94.4%) were colectomy-free, and 15 (83.3%) were in glucocorticosteroid-free remission at study end. No predictors of response were identified.

**CONCLUSION:** IFX is effective for acute severe ulcerative colitis in real-life clinical practice. Two-thirds of patients avoided colectomy, and more than 50% were in glucocorticosteroid-free remission.

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**Key words:** Ulcerative colitis; Severe; Azathioprine; Infliximab; Remission

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### INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory disorder of the gastrointestinal tract of unknown etiology, with a prevalence of between 160 and 240 per 100 000 people

in Western populations<sup>[1-3]</sup>. The condition is thought to arise from dysregulation of both the innate and adaptive immune systems, leading to an abnormal inflammatory response to commensal bacteria in a genetically susceptible individual<sup>[4]</sup>.

The clinical course of UC is characterized by periods of remission and relapse, with acute inflammatory exacerbations of disease activity which, when severe, are potentially life-threatening. The standard initial management of these inflammatory exacerbations includes high dose intravenous glucocorticosteroids in the first instance, but this strategy may be unsuccessful in up to 50% of patients<sup>[5-7]</sup>. Immunomodulating drugs such as azathioprine, whilst effective in maintaining remission<sup>[8]</sup>, act too slowly to be of use in the acute setting. Following the publication of a randomized controlled trial (RCT) by Lichtiger *et al*<sup>[9]</sup> in 1994, ciclosporin has been used as medical rescue therapy in acute severe UC, in order to avoid colectomy in the short-term, and to act as a “bridge” to long-term thiopurine therapy<sup>[10]</sup>. Several case series have since been published<sup>[11-14]</sup>, but despite response rates in the order of 50%-70%, many patients require colectomy in the longer term.

In recent years, biological therapies have emerged as a treatment option in inflammatory bowel disease (IBD). Infliximab (IFX) (Remicade®, Centocor Ortho Biotech Inc, PA, United States), a chimeric monoclonal antibody directed against human tumor necrosis factor- $\alpha$  was the first biological therapy to be approved for use in acute severe UC. The efficacy of IFX in UC has been investigated by a limited number of RCTs<sup>[15-18]</sup>. When data from all these trials were pooled in a recent meta-analysis the number needed to treat over placebo to achieve remission in one patient with moderately or severely active UC was only 4, suggesting this is a highly efficacious therapy<sup>[19]</sup>.

However, only two of these trials studied the use of IFX in acute severe UC<sup>[15,18]</sup>, one of which found no significant difference in response between IFX and placebo<sup>[18]</sup>. In addition, data from RCTs do not always translate into normal clinical practice. Data from small retrospective case series suggest that as many as one-third of patients given IFX for acute severe UC still require colectomy during the acute admission<sup>[20-23]</sup>, but larger datasets, with longer follow-up, may provide more accurate insight into the efficacy of IFX in this setting. We therefore report our 6-year experience of using IFX in acute severe UC.

## MATERIALS AND METHODS

### Participants and setting

Patients have been treated with IFX for acute severe UC since 2006 in the Leeds Gastroenterology Institute, which operates across two large teaching hospitals serving a local population of approximately 800 000, as well as receiving tertiary referrals from the surrounding area. After their first IFX infusion of 5 mg/kg in hospital,

patients attend for their second and third infusions on an outpatient basis at 2 wk and 6 wk. These are administered at a dedicated biological therapy clinic by specialist IBD nurses, who maintain a prospective database detailing demographics, response to therapy, number of infusions received, and any adverse events experienced.

All patients who received at least one dose of IFX for acute severe UC in Leeds between May 2006 and January 2012 were included. Patients were identified by cross referencing our IBD database with pharmacy records, which are accurate as all IFX infusions for inpatients are prepared in the pharmacy department. The use of IFX to treat an episode of acute severe UC was defined as need for the drug during an in-patient admission with an acute inflammatory exacerbation of disease activity. Patients receiving IFX for IBD-unclassified or pouchitis were excluded.

### Data collection

Inpatient medical records, computerized outpatient clinic letters, histopathology, endoscopy, and blood results were reviewed by one investigator. Data were collected onto a Microsoft Excel spreadsheet (XP professional edition; Microsoft, Redmond, WA, United States) designed prospectively. These included demographic details, date of UC diagnosis, extent of disease according to the Montreal classification<sup>[24]</sup>, drug therapy at time of admission, prior or current use of thiopurine or 5-aminosalicylate (5-ASA), physiological and biochemical parameters at day 0 and day 3 (mean number of stools per day, pulse, temperature, and C-reactive protein (CRP) level in mg/L), severity of UC on day 3 of their admission, according to the Travis criteria<sup>[25]</sup>, and duration of admission (in days). Total number of infusions received, any adverse events experienced (including death), requirement for colectomy, and current drug therapies were collected at discharge from the index admission, as well as at last point of follow-up.

The primary outcome of interest was colectomy-free survival, and secondary outcomes included glucocorticosteroid-free remission and safety. Patients were judged to have had an initial response to therapy if they were discharged from their index admission colectomy-free. Recording of the patient's current drug therapy at last contact allowed assessment of the achievement of glucocorticosteroid-free remission. Safety data included deaths or adverse events during the study period that were potentially related to IFX.

### Statistical analysis

The proportion of individuals who were colectomy-free at discharge from their index admission, and who were in colectomy- and glucocorticosteroid-free remission at the last point of follow-up was calculated. Demographic and clinical characteristics of those who underwent colectomy and those who were colectomy-free, both at discharge from their index admission, and during follow-up, after an initial response to IFX were compared using

**Table 1** Baseline characteristics and demographics of 44 patients with acute severe ulcerative colitis receiving infliximab *n* (%)

Characteristic	All patients ( <i>n</i> = 44)
Age at index admission <sup>1</sup>	35.7 ± 15.9
Female	16 (36.4)
Extent of disease (Montreal classification)	
E1 (limited to rectum)	2 (4.5)
E2 (distal to splenic flexure)	13 (29.5)
E3 (proximal to splenic flexure)	29 (65.9)
Current or previous smoker	18 (40.9)
Prescribed oral 5-ASA on admission	24 (54.5)
Prescribed thiopurine on admission	14 (31.8)
Median disease duration, in days, prior to first IFX infusion (IQR)	409 (16.25 to 1896.5)
First presentation of UC	12 (27.3)
Mean CRP (mg/L) on day of admission <sup>1</sup>	90.1 ± 81.9
CRP ≤ 5 on day of admission	6 (13.6)
Mean number of stools per day on admission <sup>1</sup>	12.1 ± 5.8

<sup>1</sup>Data are presented as mean ± SD. UC: Ulcerative colitis; IQR: Interquartile range; 5-ASA: 5-aminosalicylate; CRP: C-reactive protein; IFX: Infliximab.

an independent samples *t*-test for continuous variables, and Fisher's exact test for categorical variables. Multivariate logistic regression was performed in an attempt to identify independent risk factors for colectomy during the index admission, or during follow-up, controlling for all baseline demographic and clinical characteristics. All statistical analyses were performed using StatsDirect version 2.7.2 (StatsDirect Ltd, Sale, Cheshire, England), and SPSS for Windows version 19.0 (SPSS Inc, Chicago, IL, United States).

## RESULTS

Between May 2006 and January 2012, 44 patients were treated with IFX for acute severe UC. The median duration of follow-up post-first infusion was 396 d [interquartile range (IQR) 173–828 d]. There were 21 (47.7%) patients with < 1 year of follow-up, 10 (22.7%) with 1–2 years of follow-up, and 13 (29.5%) with > 2 years of follow-up post-first infusion of IFX. The mean age at presentation with acute severe UC was 35.7 years (range: 18–78 years), and mean age at diagnosis was 32.4 years (range: 13–78 years). Of the 44 patients, 16 were female (36.4%). Baseline demographic data and disease characteristics of the included patients are detailed in Table 1. All patients had abdominal X-ray performed on admission to exclude toxic megacolon, and this was repeated at the discretion of the treating physician during intravenous glucocorticosteroids. Confirmation of mucosal disease activity was obtained by flexible sigmoidoscopy.

All 44 patients met the modified Truelove and Witt criteria<sup>[26]</sup> for acute severe UC on the day of admission. Mean CRP at time of admission was 90.1 mg/L, although this was less than 5 mg/L in 6 (13.6%) patients, and mean number of stools per day on admission was 12.1. All patients received intravenous glucocorticosteroids from the time of admission for a median of 7 d

prior to IFX. There were 12 (27.3%) patients for whom the index episode of acute severe UC was their first presentation with the disease. Among the other 32 patients with an existing diagnosis of UC, 24 (75.0%) were currently receiving oral 5-ASA therapy, 14 (43.8%) were currently receiving thiopurine therapy, and a further six had previously received thiopurines but were either intolerant of them, or had experienced adverse events.

### Need for colectomy during index admission

Nine patients (20.5%) underwent colectomy during their index admission, at a median of 5 d after their first IFX infusion (range: 2–18 d). The remaining 35 patients were discharged after their first IFX infusion colectomy-free. Baseline demographic data and disease characteristics of patients according to colectomy status at discharge from hospital following the index admission are reported in Table 2.

Patients who underwent colectomy during the index admission were generally older (mean age 45.6 years versus 33.2 years), and a higher proportion were admitted with a first presentation of UC (55.6%), compared with those who were discharged without colectomy (20.0%), but these differences were not statistically significant (*P* = 0.18, and *P* = 0.09 respectively). Extent of disease, according to the Montreal classification, was not associated with need for colectomy on index admission. In terms of medication use, fewer patients who underwent colectomy were receiving oral 5-ASAs or thiopurines on admission to hospital, but only the latter difference was statistically significant (*P* = 0.04). Those who underwent colectomy had significantly higher CRP values both on admission, and at day 3, than those who avoided colectomy (*P* = 0.002, and *P* = 0.04, respectively). All nine patients who required colectomy met the Travis criteria at day 3, compared with only 15 (42.9%) of those who did not undergo surgery at the index admission (*P* = 0.002). Among those who were colectomy-free at discharge 31.4% received IFX at day 5 or sooner, compared with only 11.1% of those who underwent colectomy (*P* = 0.41). No predictors of need for colectomy during the index admission were identified by multivariate logistic regression.

### Colectomy-free survival at study end

Of the 35 patients who avoided colectomy during their index admission, 33 received standard three-dose induction with IFX. At the last point of follow-up, 29 (65.9%) of 44 patients remained colectomy-free. Thus, 82.9% (29/35) of those who responded to IFX on the index admission remained colectomy-free during follow-up. Among these 35 patients, 17 (48.6%) had < 1 year of follow-up, 8 (22.9%) had 1–2 years of follow-up, and 10 (28.6%) had > 2 years of follow-up post-first infusion of IFX. Two patients in each of these groups underwent colectomy during follow-up ( $\chi^2$  for trend, *P* = 0.69). The median time from first IFX infusion to colectomy for the six patients who had colectomy following an initial response to IFX therapy was 278 d (IQR 136.5–401.25 d).



**Table 2** Clinical characteristics and demographics of 44 patients with acute severe ulcerative colitis receiving infliximab, according to colectomy status after index admission and at last point of follow-up *n* (%)

	Colectomy status after index admission			Colectomy status at last point of follow-up		
	Colectomy during index admission ( <i>n</i> = 9)	Discharged colectomy-free ( <i>n</i> = 35)	<i>P</i> value <sup>2</sup>	Colectomy during index admission or follow-up ( <i>n</i> = 15)	Colectomy-free survival ( <i>n</i> = 29)	<i>P</i> value <sup>2</sup>
Age at index admission <sup>1</sup>	45.6 ± 24.7	33.2 ± 12.1	0.18	42.2 ± 21.4	32.4 ± 11.3	0.11
Male	7 (77.8)	21 (60.0)	0.45	10 (66.7)	18 (62.1)	1.0
First presentation of UC	5 (55.6)	7 (20.0)	0.09	6 (40.0)	6 (20.7)	0.28
Disease extent	E1: 0 (0) E2: 3 (33.3) E3: 6 (66.7)	E1: 2 (5.7) E2: 10 (28.6) E3: 23 (65.7)	0.75 <sup>3</sup>	E1: 2 (13.3) E2: 3 (20.0) E3: 10 (66.7)	E1: 0 (0) E2: 10 (34.5) E3: 19 (65.5)	0.10 <sup>3</sup>
Current or previous smoker	4 (44.4)	14 (40.0)	1.0	6 (40.0)	12 (41.4)	1.0
Prescribed oral 5-ASA on admission	3 (33.3)	21 (60.0)	0.26	8 (53.3)	16 (55.2)	1.0
Prescribed thiopurine on admission	0 (0)	14 (40.0)	0.04	3 (20.0)	11 (37.9)	0.31
CRP (mg/L): day 0 <sup>1</sup>	163 ± 62.5	71 ± 76	0.002	111.0 ± 83.0	79.0 ± 80.6	0.23
CRP (mg/L): day <sup>1</sup>	96 ± 69	39 ± 41.5	0.04	65.3 ± 65.5	42.9 ± 44.3	0.25
Number of stools per day: day 0 <sup>1</sup>	13.3 ± 5.6	11.7 ± 5.9	0.46	13.0 ± 5.7	11.6 ± 5.9	0.44
Number of stools per day: day 3 <sup>1</sup>	8.7 ± 4.6	6.5 ± 3.3	0.21	7.7 ± 4.2	6.6 ± 3.3	0.39
Met Travis criteria at day 3	9 (100)	15 (42.9)	0.002	10 (66.7)	14 (48.3)	0.34
Received IFX on day 5 or sooner	1 (11.1)	11 (31.4)	0.41			

<sup>1</sup>Data are presented as mean ± SD; <sup>2</sup>Independent samples *t*-test for continuous data and Fisher's exact test for categorical data; <sup>3</sup> $\chi^2$  for trend. UC: Ulcerative colitis; 5-ASA: 5-aminosalicylate; CRP: C-reactive protein; IFX: Infliximab.

Demographic data and clinical characteristics of those who were colectomy-free at study end and those requiring colectomy at any point during the study are reported in Table 2.

Those who were colectomy-free at end of follow-up were generally younger, more likely to have had an established diagnosis of UC prior to their index admission, more likely to be receiving thiopurines on admission, and had lower mean CRP levels at admission, and on day 3, but none of these differences were statistically significant. Again, no predictors of need for colectomy at any point during follow-up were identified by multivariate logistic regression.

There were 18 of the 35 patients who avoided colectomy during their index admission who received IFX as a "bridge" to commencement of thiopurine therapy during, or soon after, the index admission. Of these, 17 (94.4%) were colectomy-free at the end of follow-up, compared with 12 of the 17 (70.6%) who did not commence thiopurine therapy (*P* = 0.09).

### Glucocorticosteroid-free remission at study end

Of the 29 individuals who were colectomy-free at the last point of follow-up, 25 (86.2%) were in glucocorticosteroid-free remission. Therefore of the original 44 patients, 56.8% were colectomy-free and in glucocorticosteroid-free remission at the end of follow-up. Of the four patients who were colectomy-free but not in glucocorticosteroid-free remission, two had experienced a relapse of disease activity at their most recent assessment, one was receiving long-term low-dose oral glucocorticosteroids for co-existent inflammatory arthritis but was in remission clinically, and the fourth patient was still tapering the dose of glucocorticosteroids following recent index admission. Of the 18 patients who were "bridged"

to thiopurine therapy during, or soon after, the index admission 15 (83.3%) were in glucocorticosteroid-free remission at the end of follow-up.

### Safety and tolerability of IFX

During the study period one patient died from severe sepsis in the post-operative period, 2 d post-colectomy, and 20 d after a single IFX infusion. A total of eight other patients experienced adverse events with IFX. Five of these were minor, including skin rash in two patients, flushing in one patient, elevated transaminases in one patient, and pruritus in the fifth. All of these resolved without the need for discontinuation of IFX. In the other three patients the adverse events were intolerable and led to discontinuation of the drug. These included infusion reactions in two patients, and a delayed hypersensitivity reaction in the third. Of the three patients who discontinued IFX, one underwent colectomy and ileal pouch formation 15 mo after the initial IFX infusion, one was receiving low-dose glucocorticosteroids (2.5 mg prednisolone daily) in combination with methotrexate for co-existent inflammatory arthritis, and was in clinical remission as detailed above, whilst the third was colectomy-free and in glucocorticosteroid-free remission on azathioprine at the last point of follow-up.

## DISCUSSION

This study has demonstrated that IFX is an effective rescue therapy in acute severe UC. After failure of intravenous glucocorticosteroids to control the acute severe episode, 80% of patients receiving IFX avoided the need for colectomy during the index admission. Those who met the Travis criteria on day 3 and those who were not receiving thiopurine therapy on admission were more

likely to require colectomy on their index admission. Among those who responded to IFX during their index admission, 83% remained colectomy-free and, in those who were colectomy-free, glucocorticosteroid-free remission was achieved in 86%, after a median follow-up period of 396 d. Of the total cohort of patients, 57% were colectomy-free and in glucocorticosteroid-free remission at the end of follow-up. The efficacy of IFX as a “bridge” to commencing thiopurine therapy is reinforced by the finding that over 90% of those patients “bridged” to thiopurine therapy avoided subsequent colectomy. Serious adverse events, resulting in the discontinuation of IFX were rare. However, there was one post-operative death as a result of severe sepsis.

Strengths of the study include the use of our biologics database which is maintained prospectively, allowing the inclusion of data from every patient who received IFX for acute severe UC in a large tertiary referral centre. The relatively long duration of follow-up among included individuals provides valuable, real-life data on outcomes among patients with acute severe UC receiving IFX. There are some limitations of the study. We relied on data extracted from medical records and computerized outpatient clinic letters, which may not always be accurate. We did not measure improvement of disease activity using validated indices, but instead used the dichotomous outcome measures of need for colectomy and glucocorticosteroid-free remission. As the patients included in this study are from a tertiary referral centre, the data may not be generalizable to patients in other hospitals. However, the spectrum of disease is likely to be more severe in a population such as this, which may have led to an underestimate of the efficacy of IFX in this setting. Finally, although this is one of the largest retrospective single centre experiences of the use of IFX in acute severe UC reported, the absolute number of patients involved remains small, meaning that we were unable to identify any patient demographics or clinical characteristics that were independently associated with a response to IFX therapy or avoidance of colectomy during follow-up.

The results of this study are comparable with those found in an RCT conducted in Scandinavia by Järnerot *et al*<sup>[15]</sup>, in which 71% of those treated with IFX for moderately severe or severe UC avoided colectomy over 90 d. Previous retrospective studies have demonstrated similar efficacy, with between 66% and 84% of other cohorts from the United Kingdom, Denmark and Canada avoiding colectomy during their index admission<sup>[20–23]</sup>. Retrospective studies comparing IFX with ciclosporin from New Zealand and Italy found that around 80% of patients treated with IFX avoided colectomy at 3 mo, compared with 37% and 72% respectively for ciclosporin<sup>[27,28]</sup>. Recent data from the United Kingdom national IBD audit suggest that, among those who failed first line treatment with intravenous glucocorticosteroids, response rates to IFX were generally higher than those with ciclosporin<sup>[29]</sup>. One multi-centre European RCT

comparing IFX to ciclosporin head-to-head in this setting has been published recently<sup>[30]</sup>, and another United Kingdom-based trial is ongoing<sup>[31]</sup>. The European trial recruited 115 patients with acute severe UC. There was no significant difference detected in rates of response to therapy at 7 d, failure of therapy after 98 d, or colectomy rates, leading the authors to conclude that the two treatments were equivalent, and that the choice of which of these therapies to use should be guided by physician and centre experience<sup>[30]</sup>.

In the trial reported by Järnerot *et al*<sup>[15]</sup>, IFX appeared to have a more marked effect in those with less severe disease activity. Our finding that surrogate measures of severity, including a higher CRP level on day 0 and day 3, and meeting the Travis criteria at day 3, were associated with higher colectomy rates during the index admission are consistent with this. In addition, over 30% of patients who avoided colectomy during their index admission received their first IFX infusion at day 5 or sooner, compared with only 11% of those who required colectomy. Although this result did not reach statistical significance it is noteworthy, and suggests that there may be a potential benefit associated with earlier use of IFX, before the acute episode has reached its full intensity. However, these results are not supported by the findings of a multi-centre Scottish study, in which colectomy rates were higher among those treated on day 5 or sooner, compared with those treated on or after day 6<sup>[20]</sup>.

All patients in our study who underwent colectomy during the index admission were thiopurine-naïve. This is in contrast to both the Scandinavian and Scottish studies, in which thiopurine use prior to admission did not appear to affect need for colectomy<sup>[15,20]</sup>, although the numbers of patients receiving thiopurines at the time of admission in both these studies were smaller. Those who were “bridged” to thiopurine therapy in our study, following an initial response to IFX, appeared less likely to require colectomy during subsequent follow-up, although a large French multicentre case series of IFX in UC, which included patients with both acute severe and chronic relapsing disease, found that immunomodulator use was not predictive of the need for IFX optimization, IFX failure, or colectomy<sup>[32]</sup>. However, the role of thiopurines and IFX in this setting is still evolving, with preliminary results from the UC SUCCESS trial showing superiority of combination IFX and azathioprine therapy over either therapy alone in the setting of moderate to severe UC<sup>[33]</sup>.

In conclusion, this study provides further evidence for the efficacy and safety of IFX as rescue therapy in acute severe UC, in one of the largest cohorts of patients with a longer duration of follow-up than previously available from other retrospective, real-life data. Overall, 66% of patients were colectomy-free at study end, and 57% had also achieved glucocorticosteroid free-remission. Although serious adverse events were rare, the mortality rate of 2% is a reminder for clinicians of the profound effects of biological therapy on the im-

mune system, in a group of patients who are already seriously ill. The results of this study suggest that the use of IFX as a “bridge” to thiopurine therapy in patients with acute severe UC is highly effective, but even among patients who are already receiving, or are intolerant of, thiopurines the use of three-dose induction therapy with IFX may avoid the need for colectomy in a significant number.

## COMMENTS

### Background

Ulcerative colitis (UC) is a chronic inflammatory condition affecting the lower gastrointestinal tract. Acute exacerbations of inflammation are managed using intravenous glucocorticosteroids initially, but if these fail biological therapies can be used in an attempt to control inflammation and avoid the need for surgery. Infliximab (IFX) is a monoclonal antibody directed against tumour necrosis factor alpha.

### Research frontiers

Whilst randomised controlled trials have established the efficacy of IFX in UC, there have been only two placebo controlled trials specifically in the context of acute severe UC. Since results from clinical trials are not always replicated under normal clinical conditions and over longer durations of follow-up, results from real-life experience are essential to provide further insight into the efficacy of IFX in this setting.

### Innovations and breakthroughs

This single-centre review shows that, over longer follow-up periods and in real-life clinical settings, similar outcomes can be obtained to those in the original clinical trials, with two-thirds of patients avoiding the need for surgery. It also sheds light on the role of azathioprine alongside IFX therapy.

### Applications

This study will inform clinicians and patients of the likely outcomes if IFX is used as rescue therapy in acute severe UC, unresponsive to steroid treatment.

### Terminology

The use of IFX to treat an episode of acute severe UC was defined as need for the drug during an in-patient admission with an acute inflammatory exacerbation of disease activity.

### Peer review

This is a well-done paper dealing with IFX rescue treatment of patients with UC. The authors are completely right that observations from real life situations are of much higher importance than those obtained from an artificial setting of a randomized controlled trial.

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## Pegylated interferon alfa and ribavirin for children with chronic hepatitis C

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### Abstract

**AIM:** To study current treatment options for pediatric hepatitis C infection and their associated success rates.

**METHODS:** We retrospectively reviewed charts of thirty children who had been treated with combination therapy of pegylated interferon alfa plus ribavirin for chronic hepatitis C infection. Patients had been treated with ribavirin (15 mg/kg per day) and either pegylated interferon alfa 2a (180 mg/m<sup>2</sup> once weekly) or pegylated interferon alfa 2b (1.5 mg/kg once weekly). Patients' follow-up included subjective assessment of complaints, physical examination including weight and height, as well as laboratory evaluations for viral load [before treatment, at 12 wk, and 6 mo following treatment completion, as determined by sustained viral response (SVR)], complete blood count, liver enzymes, alkaline phosphatase, bilirubin, renal function tests,

and thyroid function tests. For patients not achieving a two log decrease in viral load at treatment week 12, treatment was discontinued and the patient was considered a treatment non-responder.

**RESULTS:** Thirty children aged 3-18 years were included in the study. Twenty patients (11 males, 9 females) received pegylated interferon alfa 2b and ten patients (6 males, 4 females) received pegylated interferon alfa 2a. Twenty-three patients were infected with genotype 1, six patients were infected with genotype 3, and one patient was infected with genotype 2. Twenty patients (67%) achieved SVR. Treatment success rates were 90% with pegylated interferon alfa 2a vs 55% with pegylated interferon alfa 2b. Although a trend was noted for improved outcomes in the group receiving pegylated interferon alfa 2a, there were no statistically significant outcome differences between the two treatment groups ( $P = 0.1$ ). Treatment success was 56.5% for patients infected with genotype 1 virus, compared to 100% for patients infected with other genotypes ( $P = 0.064$ ). There was no difference in treatment response between males and females. A cut-off age of twelve years was used to dichotomize younger vs older participants; however, no difference in treatment response was observed between these groups. Using multivariate regression analysis, we could not determine predictors for achieving SVR from among the variables we examined (age, sex, and viral genotype). Although we noted a trend toward SVR with peginterferon alfa-2a, there was no statistical difference between the two peginterferons. A high incidence of adverse reactions to treatment was noted. Twenty-five patients (83%) suffered from at least one adverse reaction, but most experienced more than one adverse reaction. All patients except one became leukopenic (white blood cell count less than 5500 leukocytes/ $\mu$ L), six (20%) became anemic (hemoglobin less than 110 g/L), and one (3.3%) became thrombocytopenic (platelets less than 100 000/ $\mu$ L).

**CONCLUSION:** Combination therapy to treat hepatitis

C in children is as effective as in adults. There may be a benefit for treatment with pegylated interferon alfa 2a.

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**Key words:** Hepatitis C virus; Interferon alfa; Ribavirin; Children; Sustained viral response

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## INTRODUCTION

Hepatitis C virus (HCV) infection is relatively uncommon in the pediatric population. Populations at risk of HCV infection are infants born to infected mothers who acquire the infection vertically, and children with chronic diseases who acquire the virus through infected blood products<sup>[1]</sup>. Vertical transmission is the predominant source for new pediatric HCV infections. Estimated transmission rates are 2%-5% when the mother is viremic during pregnancy<sup>[1,2]</sup>. Mothers with greater than 10<sup>6</sup> copies/mL of HCV RNA are more likely to transmit the infection to their infants compared to mothers with lower levels of viremia. Chronic hepatitis C (CHC) develops in 55%-80% of infected children<sup>[2,3]</sup>. The prevalence of HCV in children in developed countries ranges from 0.1% to 0.4%<sup>[4-6]</sup>. Treatment is contraindicated for patients less than three years of age because of safety concerns and to allow for spontaneous viral clearance. After age four, spontaneous viral clearance is unlikely<sup>[7]</sup>. The rate of viral clearance in children with CHC who acquired the infection vertically is 20%<sup>[8]</sup>. Most children who clear the virus do so during the first five years of follow-up.

In most cases, HCV infection in children is asymptomatic. Histological findings are minor and severe complications are uncommon. However, chronic hepatic inflammation from HCV infection can progress to advanced liver fibrosis and cirrhosis in four percent to six percent of infected children<sup>[9,10]</sup>. The risk of developing cirrhosis is approximately 20% and the risk of developing hepatocellular carcinoma is 2%-5%<sup>[11]</sup>. Hepatocellular carcinoma has been reported in adolescents with CHC<sup>[12]</sup>.

The current treatment of choice for CHC in children, as in the adult population<sup>[13,14]</sup>, is combination pegylated interferon alfa plus ribavirin. We have been prescribing this treatment regimen for the last seven years. We retrospectively reviewed charts of patients who had been treated for CHC with pegylated interferon alfa plus ribavirin in several Israeli Pediatric Gastroenterology Centers.

## MATERIALS AND METHODS

### Patient selection

Complete data were available for a total of thirty children

with chronic HCV infection who were treated between 2003 and 2010. Chronic HCV infection was diagnosed by the presence of anti-HCV antibodies and HCV RNA positivity. Additional available information included: alanine aminotransferase (ALT) levels during the three months prior to treatment, laboratory studies during follow-up, and, for some patients, liver biopsy specimens showing evidence of CHC or Actitest and Fibrotest results.

Ten patients were treated with pegylated interferon alfa 2a (180 mg/m<sup>2</sup> once weekly) plus ribavirin 15 mg/kg per day and 20 patients were treated with pegylated interferon alfa 2b (1.5 mg/kg once weekly) plus ribavirin (15 mg/kg per day). The decision for which peginterferon was prescribed depended on the patient's medical insurance. Length of treatment was 24 wk for patients with genotypes 2 or 3 (seven patients) and 48 wk for patients with genotype 1 (twenty-three patients). Patient follow-up included assessment of subjective complaints, physical examination with weight and height, and laboratory workup which included viral load [before treatment, at 12 wk, and 6 mo following treatment completion, as determined by sustained viral response (SVR)], complete blood count, liver enzymes, alkaline phosphatase, bilirubin, renal function tests, and thyroid function tests. In patients not achieving a two log<sub>10</sub> IU/mL decrease in their viral loads at week 12, treatment was discontinued and the patient was considered a treatment non-responder.

### Statistical analysis

Statistical analysis was performed using the SPSS software package version 15 (SPSS, Chicago, IL, United States). The normality of quantitative variables was tested using the Kolmogorov-Smirnov test. Because most of the quantitative variables were not normally distributed, the Mann-Whitney *U* test was used to analyze differences between SVR groups. Fisher's exact test was used to determine the relationship between SVR groups and categorical variables (gender, treatment type, genotype). Logistic regression was performed to predict relationships between SVR groups and several independent variables. A *P* value < 0.05 was considered statistically significant.

## RESULTS

### Patient characteristics

Thirty patients (13 girls and 17 boys) aged three to eighteen years were included in this study. Patients' characteristics and results are shown in Table 1. Twelve acquired HCV vertically, eleven through blood products, one by needle stick, and six patients had no identifiable source of infection.

It should be noted that several patients had underlying comorbidities: one patient had Becker Muscular Dystrophy, one patient had proctitis, one had Congenital Adrenal Hyperplasia, one had human immunodeficiency virus infection, and one had Obstructive Sleep Apnea. Twenty-three patients were infected with HCV genotype 1 (genotype 1b in twenty patients and genotype 1a in three patients). Six patients had genotype 3a, and one

**Table 1** Characteristics and results for non-responders and those who achieved a sustained virologic response *n* (%)

	Non-responders ( <i>n</i> = 10)	Gained SVR ( <i>n</i> = 20)	<i>P</i> value
Male	6 (60)	11 (55)	1.00
Age ≥ 12 yr	7 (70)	14 (70)	1.00
Peginterferon alfa-2a treatment	1 (10)	9 (45)	0.1
Genotype 1	10 (100)	13 (65)	0.065
WBC (cells/ $\mu$ L) <sup>1</sup>	3422.22 ± 839.47	3296.500 ± 834.57	0.39
HGB (g/L) <sup>1</sup>	110.99 ± 9.1	110.26 ± 10	0.07
ALT (U/L) <sup>1</sup>	52.33 ± 16.42	77.85 ± 41.98	0.08
AST (U/L) <sup>1</sup>	43.90 ± 19.31	55.65 ± 31.39	0.54
Weight (kg) <sup>1</sup>	48.66 ± 23.76	51.62 ± 21.96	0.66
Median viral load	747 000	332 500	0.49

<sup>1</sup>Data are presented mean ± SD. SVR: Sustained virologic response; WBC: White blood cells; HGB: Hemoglobin level; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

patient had genotype 2. Viral loads prior to treatment ranged from 134 000 to 26 200 000 copies/mL. ALT levels prior to treatment ranged from 24 to 183 U/L (mean 69.93 ± 37.64 U/L).

Liver histology ranged from mild chronic portal inflammation to moderate portal inflammation with fibrous expansion. Fibrotest scores ranged from 0 to 3 and activity ranged from 0 to 3 on the Actitest. Twenty-six patients were interferon-naïve, three patients were non-responders to previous interferon monotherapy, and one patient was a non-responder to previous pegylated interferon alfa 2a therapy (and had also been treated with pegylated interferon alfa 2b).

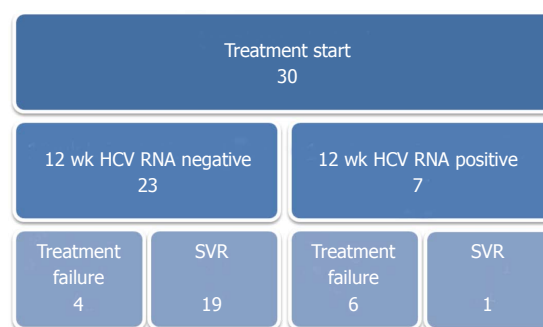
### Treatment response

Viral load at 12 wk was undetectable in twenty-two patients, slightly positive in one patient, and positive in six patients. For one patient, viral load examination was delayed and was not tested until six months after treatment initiation, at which time it was negative (Figure 1). SVR six months following treatment completion was achieved in twenty patients, for an overall treatment success rate of 67%.

Among twenty-three patients with undetectable HCV RNA [as measured by polymerase chain reaction (PCR)] following 12 wk of treatment, 19 achieved SVR. Only one of seven patients who were positive for HCV RNA at 12 wk achieved SVR. This patient's viral load had decreased greater than two log<sub>10</sub> IU/mL at 12 wk compared to baseline (Figure 1).

Forty-three percent (10/23) of those with genotype 1 did not achieve SVR; of those, four responded to treatment after twelve weeks, and six were non-responders. One patient who failed to respond was non-compliant with ribavirin, and another patient stopped ribavirin due to serious headaches and rash.

Twenty patients (eleven males, nine females) received pegylated interferon alfa 2b and ten patients (six males, four females) received pegylated interferon alfa 2a. The success rate was 90% (9/10) for those receiving pegylated interferon 2a combined with ribavirin (six patients

**Figure 1** Hepatitis C virus RNA polymerase chain reaction results 12 wk after treatment and outcomes 6 mo after treatment completion. HCV: Hepatitis C virus; SVR: Sustained viral response.

with genotype 1b, three with genotype 1a, and one with genotype 3a). The success rate for those with genotype 1b was 83% (5/6).

Fifty-five percent (11/20) of patients receiving pegylated interferon alfa 2b and ribavirin treatment (fourteen patients with genotype 1b, five patients with genotype 3a, and one patient with genotype 2) achieved SVR. Only 36% (5/14) of patients with genotype 1b achieved SVR with this treatment. Although a trend was noted in favor of pegylated interferon alfa 2a, there were no significant differences between the two treatment groups ( $P = 0.1$ ). The treatment success rate was 56.5% in patients infected with genotype 1 virus, compared to 100% in patients infected with non-1 genotypes ( $P = 0.064$ ).

There was no statistical difference in response to treatment between males and females. A cut-off age of twelve years was used to compare response to treatment between younger and older patients; no statistical difference was observed. Treatment failure occurred only among those infected with genotype 1 HCV.

We could not determine predictors for achieving SVR from among the variables examined (age, sex, and viral genotype) in our multivariate regression analysis. We did observe a trend toward greater SVR when using peginterferon alfa-2a, but this difference between the two peginterferons was not statistically significant, most likely due to our small sample.

### Adverse reactions

Adverse events were common, occurring in 83% of patients (25/30) and including flu-like symptoms, malaise, headaches, fever, lymphadenopathy, anorexia, weight loss, hair loss, myalgia, fat necrosis, somnolence, sleep disturbance, anemia, neutropenia, thrombocytopenia, oral aphthae and aseptic meningitis (following treatment completion). No patient experienced severe bone marrow suppression (Table 2).

Leukocyte counts before treatment ranged from 3630 to 11400 cells/ $\mu$ L (mean ± SD, 7540.69 ± 1950.3 cells/ $\mu$ L) and from 1750 to 5770 cells/ $\mu$ L (mean ± SD, 3335.5 ± 823 cells/ $\mu$ L) during treatment. The Mann-Whitney *U* test showed a significant difference between leukocyte levels before and during treatment ( $P < 0.001$ ). Hemoglobin levels also decreased, from 116 to 169 g/L (mean ±

**Table 2** Comparison of adverse reactions between those receiving peginterferon-alfa 2a and those receiving peginterferon-alfa 2b

Adverse reaction	Peginterferon- alfa 2a (n = 10)	Peginterferon- alfa 2b (n = 20)	Total
None	0	5	5
Flu-like symptoms	4	5	9
Weight loss	1	3	4
Hair loss	1	3	4
Headache	2	2	4
Aseptic meningitis	1	0	1
GI symptoms	1	4	5
Oral aphthae	1	0	1
Pruritus	1	0	1
Fat necrosis	1	1	2
Sleep disturbance	2	0	2
Lymphadenopathy	1	0	1
Absolute neutropenia	0	2	2
Anemia (HGB < 110 g/L)	2	4	6
Thrombocytopenia (platelets < 100 000 cells/ $\mu$ L)	1	0	1
Direct hyperbilirubinemia (mg/dL)	1	0	1
Indirect hyperbilirubinemia (mg/dL)	0	1	1

GI: Gastrointestinal; HGB: Hemoglobin level.

SD,  $135.3 \pm 12.8$  g/L) before treatment to 95 to 139 g/L (mean  $\pm$  SD,  $115 \pm 10.2$  g/L) during treatment, as determined by *t*-test ( $P < 0.001$ ).

Only one patient experienced direct hyperbilirubinemia of 15 mg/L, with a total bilirubin level of 18 mg/L. Another patient had indirect hyperbilirubinemia of 26 mg/L during treatment. All patients had normal gamma-glutamyl transferase levels and renal function throughout treatment. One patient developed hypothyroidism which subsequently resolved.

## DISCUSSION

Combination therapy of pegylated interferon alfa plus ribavirin for chronic HCV infection in children was found to be as effective as in adults. Fifty-seven percent of children infected with genotype 1 achieved SVR and children infected with other HCV genotypes achieved 100% SVR. The success rates are similar to success rates reported in other pediatric studies<sup>[13,15]</sup>.

Treatment strategies for CHC have evolved over the years, from interferon monotherapy to combination interferon alfa plus ribavirin<sup>[16]</sup> to combination pegylated interferon alfa plus ribavirin<sup>[14,15,17,18]</sup>. This latter combination is currently the treatment of choice for CHC in children, as in adults<sup>[13,14]</sup>. Schwarz *et al.*<sup>[15]</sup> reported that combination ribavirin plus peginterferon alfa is superior to peginterferon alfa and placebo for children and adolescents with CHC. Two options exist for pegylated interferon alfa therapy: pegylated interferon alfa 2a and pegylated interferon alfa 2b<sup>[19-23]</sup>. The main treatment goal is to achieve an SVR, defined as undetectable serum HCV RNA for six months following cessation of treatment. Treatment outcomes depend on HCV genotype and viral

load at the beginning of treatment<sup>[13,17,24]</sup>. The genotype is a key predictor of treatment response<sup>[24]</sup>. Wirth *et al.*<sup>[14]</sup> found that patients infected with genotype 1 experienced 53% SVR, compared to patients with genotypes 2 or 3 who had 93% SVR, and patients with genotype 4 who had 80% SVR. Baseline viral load is the main response predictor for patients infected with HCV genotype 1. SVR may be more likely in patients who have lower viral loads<sup>[14]</sup>. In a systematic review, Hu *et al.*<sup>[13]</sup> reported that SVR rates in children with CHC ranged from 30% to 100%, comparable to those seen among adults.

Combination treatment causes high rates of adverse reactions, with almost all children suffering from transient flu-like symptoms. Other adverse reactions are diverse. In our study, 83% of patients suffered from adverse reactions, but almost all patients remained compliant with therapy. Indeed, in most studies, the treatment is tolerated and compliance is good<sup>[24,25]</sup>. The treatment protocol has been associated with significant changes in body weight, linear growth and body composition (loss of fat mass); however, these effects seem reversible<sup>[26]</sup>.

In adults, HCV RNA PCR results after twelve weeks of treatment predict treatment outcomes. Failure to respond (a less than two log<sub>10</sub> drop from baseline HCV RNA levels) is associated with non-response to treatment, and the therapy should be discontinued. It is unknown whether this rule applies to pediatric patients as well<sup>[13]</sup>. However, this rule does fit with our current findings.

All treatment failures in our study occurred in patients infected with genotype 1, which was also the most frequent genotype among our patients. SVR rates depend on genotype, as in the adult population, and success rates are significantly better (greater than 90%) in patients infected with genotypes 2 and 3 compared to patients infected with genotype 1 or 4 (approximately 50%)<sup>[14]</sup>.

Although both pegylated interferon alfa regimens have similar safety profiles, success rates differ. In a systematic review of twelve randomized clinical trials including 5008 patients, Awad *et al.*<sup>[20]</sup> concluded that peginterferon alfa-2a is associated with higher SVR than peginterferon alfa-2b. We have also noted different success rates between the two pegylated interferon alfa products, as reported in previous studies<sup>[19,20,22,23]</sup>. With caution and consideration of our small sample size, our results show the trend by which combination treatment with pegylated interferon alfa 2a may be superior to pegylated interferon alfa 2b. Such a trend has been shown previously only in adult studies<sup>[19,20,22]</sup>.

The main weakness of the study is its retrospective nature and relatively small number of patients. Nevertheless, our results are consistent with previous studies in children and adults and our study is the first to compare the two pegylated interferon products in children. Further prospective studies are highly encouraged.

## COMMENTS

### Background

Hepatitis C virus (HCV) is a virus that chronically infects the liver. Infection by



this virus is a known risk factor for liver disease, liver failure and its complications, and even cases of liver tumors (hepatocellular carcinoma). There are limited publications about experience in treating children, recommendations for a preferred regimen, and data on treatment success rates. In this study, thirty children with chronic hepatitis C had been treated with combination therapy of peginterferon alfa plus ribavirin (as is recommended in adults). Ten of them had been given peginterferon alfa 2a, twenty of them had been given peginterferon alfa 2b, since there is no accepted preference of either of these drugs.

### Research frontiers

This paper introduces the Israeli experience in treatment of children with chronic HCV infection, including success rates and adverse reactions.

### Innovations and breakthroughs

The study found that success rates in children are very similar to those reported in adults. Although no significant superiority of either of the drugs was found, (most probably due to small group size), a trend for better results was noted with peginterferon alfa 2a. Even though side effects were very common during the treatment regimen, children were found to be very compliant, and most of them completed the treatment course.

### Applications

The study results suggest that success rates of treatment are similar to those noted in adults, and that treatment with combination peginterferon alfa 2a plus ribavirin may be superior.

### Terminology

Peginterferon alfa: A pegylated interferon drug that is given in a subcutaneous manner once a week, as opposed to previous treatment with interferon, which was given by injections 3 times a week. Treatment success: Achieving sustained viral response means that six months after treatment completion there is no detectable HCV RNA in the blood.

### Peer review

Data on HCV therapy in pediatric patients are limited, thus this paper adds important information.

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## Granulocyte-colony stimulating factor therapy improves survival in patients with hepatitis B virus-associated acute-on-chronic liver failure

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### Abstract

**AIM:** To evaluate the safety and efficacy of granulocyte-colony stimulating factor (G-CSF) therapy in patients with hepatitis B virus (HBV)-associated acute-on-chronic liver failure (ACLF).

**METHODS:** Fifty-five patients with HBV-associated ACLF were randomized into two groups: the treatment group and the control group. Twenty-seven patients in the treatment group received G-CSF (5 µg/kg per day, six doses) treatment plus standard therapy, and 28 patients in the control group received standard therapy only. The peripheral CD34<sup>+</sup> cell count was measured consecutively by flow cytometry. Circulating

white blood cell count, biochemical parameters, and other clinical data of these patients were recorded and analyzed. All patients were followed up for a period of 3 mo to evaluate the changes in liver function and survival rate.

**RESULTS:** The peripheral neutrophil and CD34<sup>+</sup> cell counts in the G-CSF group increased on day 3 from the onset of therapy, continued to rise on day 7, and remained elevated on day 15 compared to those of the control group. Child-Turcotte-Pugh score of patients in the treatment group was improved on day 30 from the onset of G-CSF therapy, compared to that in the controls ( $P = 0.041$ ). Model for End-Stage of Liver Disease score of patients in the treatment group was improved on day 7 ( $P = 0.004$ ) and remained high on day 30 from the onset of G-CSF therapy ( $P < 0.001$ ) compared to that in controls. After 3 mo of follow-up observation, the survival rate in the treatment group (48.1%) was significantly higher than that in the control group (21.4%) ( $P = 0.0181$ ).

**CONCLUSION:** G-CSF therapy promoted CD34<sup>+</sup> cell mobilization in patients with HBV-associated ACLF, and improved the liver function and the survival rate of these patients.

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**Key words:** Acute-on-chronic liver failure; Granulocyte-colony stimulating factor; Hepatitis B virus

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## INTRODUCTION

Hepatitis B virus (HBV) infection is one of the major public health problems. It is estimated that over 350 million individuals are chronically infected with HBV worldwide<sup>[1]</sup>. Some of these patients develop severe liver diseases such as acute-on-chronic liver failure (ACLF), liver cirrhosis, and hepato-cellular carcinoma<sup>[2]</sup>. The mortality rate of HBV-associated ACLF varies between 70%-80%<sup>[3,4]</sup>. Liver transplantation is the only effective treatment currently available, but its application is limited by the shortage of donors and the high cost.

In order to overcome these problems, alternative approaches have been proposed, such as an artificial liver support system<sup>[5]</sup>, liver cell transplantation<sup>[6]</sup> and stem cell transplantations<sup>[7]</sup>. In particular, the great potential of stem cells to differentiate into multiple cell lineages raises the exciting hypothesis that these cells can be used in tissue repair and tissue-specific cell regeneration, when tissue-resided stem cells are not sufficient for the regeneration of a failing organ.

During liver regeneration, bone marrow-derived hematopoietic stem cells (HSC) may mobilize to the liver and, together with hepatocytes and intrahepatic stem cells, contribute to the proliferation of liver cells<sup>[8]</sup>. Hepatocytes carrying a Y chromosome were detected in the livers of female recipients who had received bone marrow transplantation from male donors<sup>[9]</sup>. Granulocyte-colony stimulating factor (G-CSF) can be used to mobilize stem cells to the periphery in patients with advanced liver disease, or to promote adequate numbers of cells for further transplantation. The safety and efficacy of this protocol has been investigated<sup>[10]</sup>. In experimental animal models, G-CSF ameliorated liver injury and improved the survival rate in rats with D-galactosamine-induced acute liver failure<sup>[11,12]</sup>. When administered to patients with severe liver cirrhosis, G-CSF boosted the numbers of peripheral leukocytes and CD34<sup>+</sup> bone marrow-derived HSCs<sup>[13]</sup>. Therefore, G-CSF therapy may be beneficial for liver regeneration in patients with different kinds of liver injuries.

Our objective in this clinical study was to evaluate the effects of G-CSF therapy on the proliferation of peripheral CD34<sup>+</sup> cells and on liver function in patients with HBV-associated ACLF. The parameters of liver function in these patients were consecutively measured. Child-Turcotte-Pugh (CTP) score, Model for End Stage of Liver Disease (MELD) score and survival rate of these patients were evaluated during a 3-mo follow-up study.

## MATERIALS AND METHODS

### Patients

A total of 55 patients with HBV-associated ACLF were recruited to our center from June 2009 to May 2011. HBV-associated ACLF, defined by the Asian Pacific Association for the Study of the Liver Working Party<sup>[2]</sup>, is an acute hepatic insult manifested as jaundice (serum bilirubin  $\geq 5$  mg/dL) and coagulopathy [international

normalized ratio (INR)  $\geq 1.5$  or prothrombin activity  $< 40\%$ ], with complications of ascites and/or encephalopathy within 4 wk in patients previously diagnosed or undiagnosed with chronic liver disease. Other inclusion criteria included: (1) the presence of hepatitis B surface antigen in the serum for at least 6 mo; (2) the evidence of active viral replication as indicated by detectable HBV DNA in the serum ( $\geq 1 \times 10^4$  copies/mL); (3) flares of hepatitis, marked by increased serum alanine aminotransferase (ALT) level to more than 5-fold of the upper limit of normal value; and (4) age between 18 to 65 years.

The exclusion criteria included the following: (1) super-infection or co-infection with hepatitis A, C, D, E, Epstein-Barr virus, cytomegalovirus, or human immunodeficiency virus; (2) a previous course of any antiviral, immuno-modulator or cytotoxic/immunosuppressive therapy for chronic hepatitis within the prior 12 mo; (3) evidence of decompensated liver disease prior to the enrollment; (4) hepato-cellular carcinoma diagnosed by computed tomography or magnetic resonance imaging; (5) co-existence of any other serious medical illnesses or other liver diseases such as autoimmune hepatitis, alcoholic liver disease, drug-induced liver injury or Wilson's disease; (6) any concurrent evidence of sepsis; (7) malignant jaundice induced by obstructive jaundice and hemolytic jaundice; and (8) prolonged prothrombin time due to blood system disease.

### Groups

This was a randomized, controlled, and double-blinded study. The sample size was determined as follows: Based on the hypothesis that G-CSF therapy can improve survival rate by 10% in the treatment group compared to the control group, with a power of 95% and an alpha error of 5%, the number of patients should be 25 in each group. A randomization number code was generated for each patient. Based on this code, each patient was assigned to receive G-CSF therapy plus standard medical therapy (G-CSF group), or standard medical therapy alone (control group). Both the patients and the investigators were blinded to the treatment regimen. The patients in the G-CSF treatment group received G-CSF (SL Pharm, Beijing, China) subcutaneously at the dosage of 5  $\mu$ g/kg per day for six consecutive days, and were monitored with daily physical examination and laboratory tests. All patients received entecavir (0.5 mg/d, Squibb Pharmaceuticals Ltd., Shang Hai, China) and standard therapy (including reduced glutathione, glycyrrhizin, ademetionine, polyene phosphatidylcholine, alprostadil, and human serum albumin) on the day of admission. The white blood cell (WBC) counts were assessed twice per week in the first two weeks. In addition, abdominal ultrasound examination was performed on days 1 and 7 to evaluate the diameters of spleen and the hepatic portal vein.

### Quantification of peripheral CD34<sup>+</sup> cells

CD34<sup>+</sup> cells in the peripheral venous blood were measured consecutively twice per week in the first two weeks



**Table 1** Characteristics of patients with hepatitis B virus-associated acute-on-chronic liver failure at the time of admission

Parameters	G-CSF group	Control group	P value
Gender (male %)	22 (81.5)	22 (78.6)	0.755
Age (yr)	43.5 (29-63)	45.9 (22-65)	0.332
WBC ( $10^9/L$ )	$5.79 \pm 1.81$	$6.61 \pm 1.71$	0.443
Neutrophil ( $10^9/L$ )	$3.53 \pm 1.46$	$3.82 \pm 1.17$	0.114
Platelets ( $10^9/L$ )	182 (147-215)	174 (149-175)	0.680
ALT (U/L)	276 (197-801)	252 (189-1239)	0.430
AST (U/L)	246 (195-788)	251 (187-980)	0.544
Total bilirubin ( $\mu\text{mol/L}$ )	336 (181-519)	320.0 (174.5-519.8)	0.605
Cr ( $\mu\text{mol/L}$ )	$83.8 \pm 16.9$	$85.4 \pm 53.87$	0.475
INR	$2.11 \pm 0.28$	$2.34 \pm 0.34$	0.606
ALB (g/L)	$29.11 \pm 4.05$	$28.75 \pm 4.63$	0.596
HBV DNA ( $\log_{10}$ )	$5.11 \pm 1.37$	$5.55 \pm 1.59$	0.280
CTP score	$12.17 \pm 1.47$	$12.25 \pm 1.29$	0.349
MELD score	$25.11 \pm 3.30$	$26.30 \pm 4.12$	0.588

Values are shown as mean  $\pm$  SD. WBC: White blood cell; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Cr: Creatine; INR: International normalized ratio; ALB: Albumin; CTP: Child-Turcotte-Pugh; MELD: Model for end stage of liver disease; HBV: Hepatitis B virus; G-CSF: Granulocyte-colony stimulating factor.

in all patients. Briefly, small aliquots of peripheral blood were incubated with PE-conjugated anti-CD34 monoclonal-antibody (BD Company, United States) for 30 min on ice. Erythrocytes were lysed with ammonium chloride for 10 min at room temperature. Cells were washed with phosphate-buffered saline, and kept on ice till flow cytometric analysis.

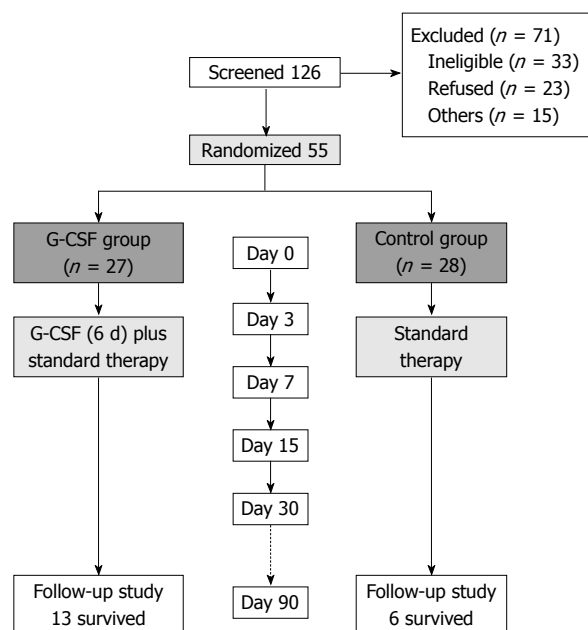
### Follow-up scheduling

All patients had daily follow-ups and physical examinations in the first month, and then at least weekly for the next 2 mo. During the 3-mo follow-up period, patients were monitored for the following parameters: the levels of serum bilirubin and albumin, prothrombin time and concentration, INR, the levels of ALT and aspartate aminotransferase, the levels of blood urea and serum creatinine, complete blood analysis, estimation of the degree of ascites, CTP score, MELD score, and hepatic encephalopathy. The survival rates were evaluated over a period of 3-mo.

Sera from patients were collected and direct polymerase chain reaction (PCR) sequencing was used to screen for HBV reverse transcriptase (RT) domain if serum HBV DNA tests were positive after patients received entecavir therapy. The *HBV* gene fragment (nt 54-1278) encompassing the complete *RT* gene (nt 130-1161) was amplified by nested PCR. The primers and reaction conditions have been described previously<sup>[14]</sup>. Substitutions at positions rt180, rt184, rt202, rt204 and rt250 were taken as resistance mutations for analysis.

### Ethics

The protocol of our study is in compliance with the ethical guidelines of the 1975 Declaration of Helsinki, and was approved by the Clinical Research Ethics Committee of the Beijing 302 Hospital. All patients or their



**Figure 1** Study design and flow algorithm of patient selection. G-CSF: Granulocyte-colony stimulating factor.

relatives in the G-CSF treatment group provided written informed consent prior to the enrollment.

### Statistical analysis

Data were compiled using Excel XP and processed using Statistical Package for Science and Society (SPSS) version 12.0 (SPSS Inc., Chicago, IL). All quantitative variables were presented as mean  $\pm$  SD. All qualitative data were described as frequency or percentage. Comparisons between groups for qualitative data were carried out using  $\chi^2$  test, Fischer's exact test, or McNemar test when appropriate. Independent sample *t* test and paired sample *t* test were used for quantitative variables with normal distribution, whereas non-parametric Mann-Whitney test and Wilcoxon signed-rank test were used for quantitative variables with non-normal distribution. In all tests,  $P < 0.05$  were considered as statistically significant.

## RESULTS

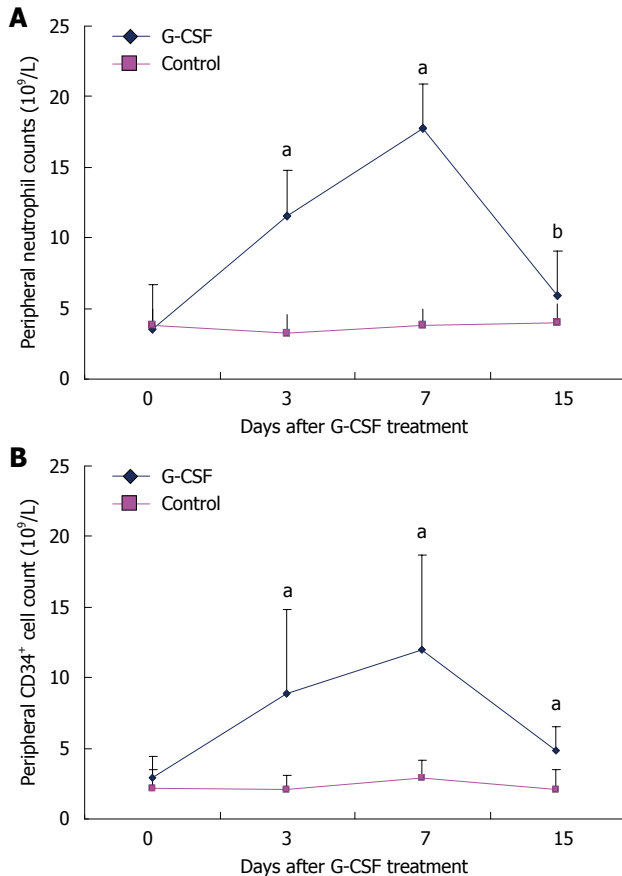
### Baseline conditions

Of the 126 patients screened, 71 were excluded and 55 patients enrolled in our study. Among them, 27 were randomized to receive G-CSF therapy, and the other 28 were included as controls (Figure 1).

The two groups showed no statistical differences in gender, age, the baseline values of peripheral WBC, platelets, and other parameters (Table 1). All patients had a history of chronic hepatitis B, and had not received anti-viral therapy prior to the enrollment.

### Peripheral neutrophil counts and CD34-positive cell counts

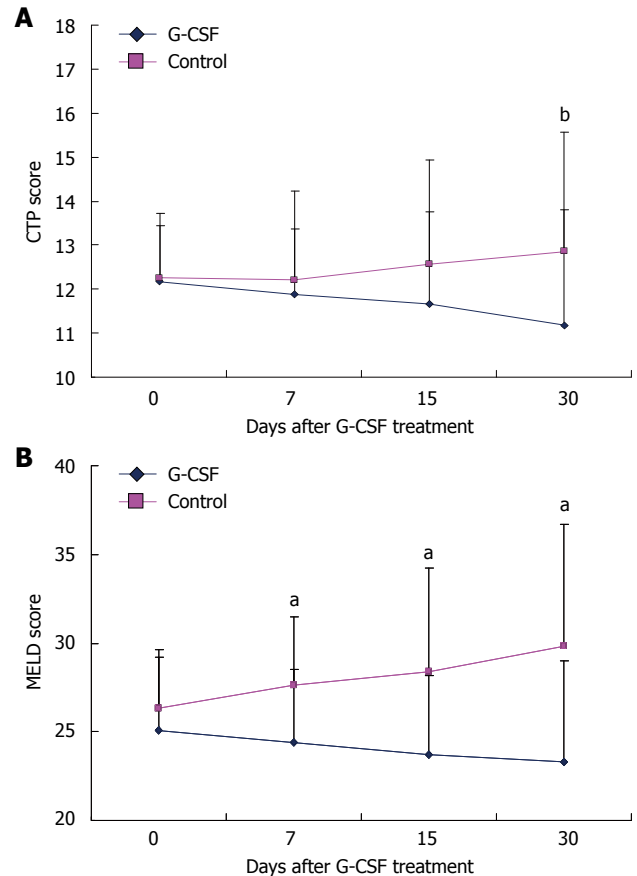
The baseline value of the circulating neutrophil count was compared between the G-CSF and the control groups



**Figure 2** Kinetics of peripheral neutrophil count (A) and CD34<sup>+</sup> cell count (B) in patients treated with granulocyte-colony stimulating factor and controls. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs controls. G-CSF: Granulocyte-colony stimulating factor.

(Table 1). No statistical difference was found between the baselines of the two groups ( $P = 0.443$ ). However, on day 3 from the onset of G-CSF therapy, the peripheral neutrophil count increased to  $(11.59 \pm 6.40) \times 10^9/L$ , significantly higher than that of the control group  $(3.29 \pm 1.25) \times 10^9/L$  ( $P < 0.001$ ). It continued to rise on day 7 to  $(17.76 \pm 10.07) \times 10^9/L$  in the G-CSF group, significantly higher than  $(3.82 \pm 1.17) \times 10^9/L$  in the control group ( $P < 0.001$ ). On day 15, the neutrophil count decreased to  $(5.88 \pm 3.69) \times 10^9/L$  in the G-CSF group, but was still higher than  $(4.02 \pm 1.33) \times 10^9/L$  in the control group ( $P = 0.032$ ) (Figure 2A).

Prior to G-CSF therapy, circulating CD34<sup>+</sup> cell counts were comparable in the two groups -  $(2.97 \pm 1.52) \times 10^6/L$  in the G-CSF group and  $(2.23 \pm 1.29) \times 10^6/L$  in the control group ( $P = 0.085$ ). On day 3 of treatment, circulating CD34<sup>+</sup> cell counts in the G-CSF group increased to  $(8.96 \pm 5.97) \times 10^6/L$ , compared to  $(2.09 \pm 1.02) \times 10^6/L$  in the control group ( $P < 0.001$ ). On day 7, the circulating CD34<sup>+</sup> cell counts continued to rise to  $(12.05 \pm 6.70) \times 10^6/L$ , compared to  $(2.97 \pm 1.22) \times 10^6/L$  in the control ( $P < 0.001$ ). On day 15, the CD34<sup>+</sup> cell counts decreased to  $(4.92 \pm 1.63) \times 10^6/L$  in the G-CSF treatment group, still significantly higher than that in the control group  $(2.11 \pm 1.39)$  ( $P < 0.001$ ) (Figure



**Figure 3** Changes in Child-Turcotte-Pugh score (A) and Model for End stage of Liver Disease score (B) in patients treated with granulocyte-colony stimulating factor and control groups. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs controls. G-CSF: Granulocyte-colony stimulating factor; CTP: Child-Turcotte-Pugh; MELD: Model for End Stage of Liver Disease.

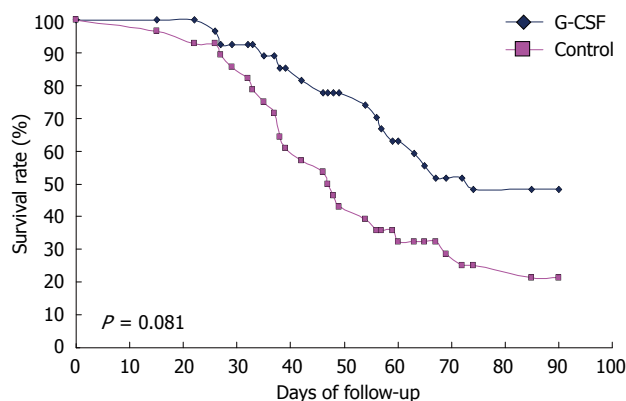
2B). On day 30, the CD34<sup>+</sup> cell counts were nearly at the same level between the two groups.

In the course of the G-CSF therapy, all patients demonstrated good tolerance. Some patients were affected by minor side effects, such as fever (8 cases), headache (5 cases), and nausea (4 cases), but all the side effects resolved within 5 d after the withdrawal of G-CSF treatment.

### Changes of liver function after G-CSF therapy

Prior to the G-CSF therapy, the CTP score was  $12.17 \pm 1.47$  in the G-CSF group, and  $12.25 \pm 1.29$  in the control group ( $P = 0.841$ ). The CTP score decreased gradually after the G-CSF therapy, and reached  $11.17 \pm 2.76$  on day 30 of treatment in the G-CSF group, compared to  $12.86 \pm 2.63$  in the control group ( $P = 0.041$ ) (Figure 3A).

The MELD score demonstrated an early decrease in the G-CSF group (Figure 3B). On day 7 of the therapy, the MELD score decreased to  $24.4 \pm 3.9$  in the G-CSF group, compared to  $27.6 \pm 4.1$  in the control group ( $P = 0.004$ ). On day 15, the MELD score was  $23.7 \pm 5.8$  in the G-CSF group, compared to  $28.4 \pm 4.5$  in the control group ( $P < 0.001$ ). This trend continued during the 30-d observation period ( $23.3 \pm 6.9$  vs  $29.8 \pm 5.7$ ) ( $P < 0.001$ ).



**Figure 4** Survival rate of patients treated with granulocyte-colony stimulating factor and in controls. G-CSF: Granulocyte-colony stimulating factor.

### Levels of serum HBV DNA and drug-resistant HBV variant monitoring

All patients enrolled in our study received entecavir antiviral treatment throughout the course of the study. After 3 mo of treatment, all the surviving patients demonstrated effective antiviral results. Of the 19 surviving patients, 16 (11 from G-CSF group, and 5 from control group) were detected negative for serum HBV DNA (below  $10^2$  copies/mL), and 3 (2 from G-CSF group and 1 from control group) were positive, but the concentrations of HBV DNA were all below  $10^3$  copies/mL. Serum HBV mutations were determined by direct sequencing methods and an entecavir-resistant mutation was not detected in these patients. No serious adverse effects of entecavir were observed in all patients.

### Survival rate

The patients' survival rates were evaluated at the end of 3 mo (Figure 4). In the G-CSF group, 13 of 27 patients survived, but only 6 of 28 patients survived in the control group ( $\chi^2$  value 5.584,  $P = 0.0181$ ). All the patients had complications of ascites and electrolyte disturbances. In the treatment group, 4 patients died of encephalopathy, 5 of gastric intestinal bleeding, 2 of hepato-renal syndrome (HRS), and 3 of sepsis. In the control group, 6 died of encephalopathy, 3 of gastric intestinal bleeding, 6 died from HRS, and 7 of sepsis. More patients in the control group died of sepsis and HRS compared to those in the G-CSF group ( $\chi^2$  value 4.863,  $P = 0.027$ ).

## DISCUSSION

In China, HBV-associated ACLF accounts for 85%-95% of all liver failure. Approximately 60% of the patients die from complications due to the lack of early liver transplantation<sup>[15,16]</sup>. Increasing evidence indicates that stem cells contribute to hepatic regeneration, which is essential for the restoration of liver function and thus the survival of the patients. Stem cells have been shown to induce the repairing process after acute liver injuries<sup>[10,12]</sup>. Therefore, G-CSF therapy may be a promising approach in the clinical treatment of patients with ACLF.

Some previous findings on the efficacy of G-CSF in patients with liver failure remain controversial. Di Campli *et al.*<sup>[17]</sup> investigated the safety and efficacy of G-CSF in patients with ACLF. They found an increased number of CD34<sup>+</sup> cells in the peripheral blood, down-regulated expression of C-X-C chemokine receptor type 4, very late activation of antigen 4 and vascular endothelial growth factor receptor in the G-CSF treatment group, but they did not show the effect of G-CSF on the survival rates of these patients. Similarly, Spahr *et al.*<sup>[18]</sup> investigated the efficacy of G-CSF in patients with alcoholic steatohepatitis, and observed an elevated peripheral CD34<sup>+</sup> cell count and proliferating hemopoietic cells in the liver tissues, although they failed to demonstrate the improvement of liver function. Recently Garg *et al.*<sup>[19]</sup> also investigated the efficacy of G-CSF in ACLF patients with promising results. Sixteen of the 23 patients in the G-CSF group survived for 60 d, compared to merely 7 of the 24 in the control group. The discrepancies between the results by different groups may be attributable to the background of the enrolled patients. For example, Di Campli enrolled patients with alcohol liver failure, whereas Garg recruited patients with various types of liver diseases, including alcoholic-related liver diseases and HBV-associated ACLF.

In our current study we selected patients with HBV-associated ACLF for investigation. This is the first report to apply G-CSF therapy to patients with chronic HBV infection. All the patients were positive for serum HBV DNA and received entecavir antiviral treatment after enrollment. The two groups showed no statistically significant differences in gender, WBC count, CTP score and MELD score prior to the onset of the G-CSF therapy.

We observed that G-CSF therapy increased the peripheral neutrophil count and CD34<sup>+</sup> cell count in patients with HBV-associated ACLF. In addition, the G-CSF treatment group demonstrated improved liver function compared to the control group, as demonstrated by the CTP and MELD scores. Our finding on survival rate was in consistency with that of Garg *et al.*<sup>[19]</sup>.

It is plausible to speculate that CD34<sup>+</sup> HSC migrated from the bone marrow to the liver, and contributed to the regeneration of the liver. Future studies, such as liver biopsy or autopsy, may provide evidence for the mobilization of the stem cells. In these patients, MELD score improved sooner than CTP score after G-CSF therapy, but the mechanism is yet to be elucidated.

Currently, there are two classical pathways for stem cells to travel from the bone marrow to the liver. The first is G-CSF mobilization. Secondly, CD34<sup>+</sup> cells can be isolated from bone marrow, purified with magnetic columns, and then re-injected into the liver through the hepatic artery or the portal vein<sup>[13,20]</sup>. However, patients with ACLF often have coagulation disorders; therefore, it is risky to apply the aforementioned complicated procedures. It is a reasonable argument that the approach of G-CSF administration is probably easier and less risky to implement than the separation, purification, and autologous transplantation of bone marrow-derived HSC cells to ACLF.

patients. Furthermore, it has been reported that contamination by red blood cells during CD34<sup>+</sup> cell isolation can impair the efficacy of autologous HSC therapy<sup>[21]</sup>.

Antiviral therapy has been deemed as an important therapy for patients with HBV-associated ACLF<sup>[2,22]</sup>. Previously, Huang *et al.*<sup>[16]</sup> reported that antiviral therapy decreased the mortality rate in patients with HBV-associated ACLF, but the patients recruited to their study had lower ALT levels and higher prothrombin activity (over 30%), which may explain the different results between these two studies. In our study, the mortality rate in the control group was higher (78.6%) than those reported in the literature. This may be attributable to several factors. Firstly, the patients in our study had larger MELD scores, of 25 to 29, indicating more serious conditions. Secondly, most patients enrolled in our study had middle-stage (PA between 20%-30%) or late-stage ACLF (PA below 20%) according to the standards of the Chinese classification system<sup>[23]</sup>, which may contribute to an increased mortality rate. Thirdly, antiviral therapy can suppress HBV replication; however, there may not be enough time for HBV suppression and liver function recovery to occur due to the short life expectancy in ACLF patients. A similar phenomenon was also observed by other researchers<sup>[24]</sup>. We also consider the possibility of HBV viral breakthrough in these patients. Serum HBV DNA mutations were determined by a direct sequencing method when serum HBV DNA was positive in patients, but we did not find any entecavir-resistant mutations. Although we did not find poor compliance in these patients, we could not exclude the possibility. In our study, all the patients received antiviral therapy to help mitigate additional hepatic insult and even liver failure caused by the re-activation of HBV.

Patients treated with G-CSF not only demonstrated a significantly better 90 d survival rate, CTP and MELD scores, but were also less likely to develop HRS and sepsis compared to the controls. This could be explained by increased numbers of neutrophils in these patients. Neutrophil dysfunction has been shown to cause sepsis and further the development of HRS in patients with ACLF<sup>[25]</sup>. We did not find a correlation between CD34<sup>+</sup> cell number and liver function improvement, which means cell numbers, micro-environment of liver tissue and other possible factors may work together to improve liver function. That mechanism needs to be further investigated.

In conclusion, this randomized, controlled study clearly demonstrated the clinical safety and efficacy of G-CSF therapy in patients with HBV-associated ACLF. The convenience of administration makes G-CSF therapy readily implementable in large-scale, multi-center clinical sites, and further supports its benefits in restoring hepatic function and improving survival rate.

## COMMENTS

### Background

Hepatitis B virus-associated acute-on-chronic liver failure (HBV-ACLF) is as-

sociated with a high mortality. Liver transplantation could significantly improve the survival rate, but is limited by many factors, especially donor shortages. To overcome these problems, alternative approaches have been proposed. Granulocyte-colony stimulating factor (G-CSF) can be used to mobilize stem cells from bone marrow to the periphery, and then to the liver, in patients with advanced liver disease, and promote liver regeneration.

### Research frontiers

In experimental animal models, G-CSF ameliorated liver injury and improved survival rate in rats with D-galactosamine-induced acute liver failure. When administered to patients with severe liver cirrhosis, G-CSF boosted the numbers of peripheral leukocytes and CD34<sup>+</sup> bone marrow-derived hematopoietic stem cells. Therefore, G-CSF therapy may be beneficial for liver regeneration in patients with different kinds of liver injuries. The safety and efficacy of a G-CSF protocol has been investigated but the efficacy is still controversial in patients with liver diseases.

### Innovations and breakthroughs

In the current study, the authors selected patients with HBV-associated ACLF for investigation. This is the first randomized trial to apply G-CSF therapy to patients with chronic HBV infection. The authors observed that G-CSF therapy increased peripheral neutrophil count and CD34<sup>+</sup> cell count in patients with HBV-associated ACLF. In addition, the G-CSF treatment group demonstrated improved liver function and survival rate compared to the control group.

### Applications

HBV-associated ACLF patients treated with G-CSF not only demonstrated a significantly better 90 d survival rate, and improved CTP and MELD scores, but were also less likely to develop HRS and sepsis compared to the controls. The convenience of administration makes G-CSF therapy readily implementable in large-scale, multi-center clinical sites to further support its benefits in restoring hepatic function and improving survival rate.

### Terminology

HBV-associated ACLF, defined by the Asian Pacific Association for the Study of the Liver Working Party, is an acute hepatic insult manifested as jaundice (serum bilirubin  $\geq 5$  mg/dL) and coagulopathy (international normalized ratio  $\geq 1.5$  or prothrombin activity  $< 40\%$ ), with complications of ascites and/or encephalopathy within 4 wk in patients previously diagnosed or undiagnosed with chronic liver disease.

### Peer review

This is an interesting study in patients with decompensated hepatitis B patients.

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## Risk factors for hepatic decompensation in patients with primary biliary cirrhosis

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### Abstract

**AIM:** To examine the clinical features and analyze prognostic factors in a prospective study of primary biliary cirrhosis (PBC) patients.

**METHODS:** From 1995 to 2010, PBC patients without hepatic decompensation seen at the Peking Union Medical College Hospital were enrolled. Clinical signs and manifestations (pruritus, persistent fatigue, jaundice and pain in the right hypochondrium), laboratory parameters (auto-antibodies for autoimmune hepatic disease, biliary and hepatic enzymes, immunoglobulin, bilirubin, and albumin) and imaging findings were recorded at entry and at specific time points during follow-up. Cox regression and Kaplan-Meier analyses, respectively, assessed the risk factors for hepatic decompensation and survival.

**RESULTS:** Two hundred and sixty-two PBC patients

were enrolled with a median follow-up of 75.2 mo (range, 21-201 mo). The 240 patients were aged  $51.5 \pm 10.2$  years at diagnosis and 91.6% were female. Two hundred and forty-five (93.5%) were seropositive for anti-mitochondrial antibodies. At presentation, 170 patients (64.9%) were symptomatic, while 96 patients (36.6%) had extra-hepatic autoimmune disease. During the follow-up period, 62 (23.7%) patients developed hepatic decompensation of whom four underwent liver transplantation and 17 died. The cumulative survival rate and median survival time were 83.9% and 181.7 mo, respectively. Cox regression analysis revealed that an incomplete ursodeoxycholic acid (UDCA) response or inconsistent treatment [ $P < 0.001$ ; hazard risk (HR) 95%CI = 2.423-7.541], anti-centromere antibodies (ACA) positivity ( $P < 0.001$ ; HR 95%CI = 2.516-7.137), alanine aminotransferase ratio (AAR) elevations ( $P < 0.001$ ; HR 95%CI = 1.357-2.678), and histological advanced liver disease ( $P = 0.006$ ; HR 95%CI = 1.481-10.847) were predictors of hepatic decompensation. The clinical features and survival of PBC in China are consistent with those described in Western countries.

**CONCLUSION:** Incomplete UDCA response or inconsistent treatment, ACA positivity, AAR elevations, and advanced histological stage are predictors of decompensation.

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**Key words:** Primary biliary cirrhosis; Risk factor; Hepatic decompensation; Survival; Ursodeoxycholic acid response; Anti-centromere antibodies; Histological stage

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## INTRODUCTION

Primary biliary cirrhosis (PBC) is a chronic cholestatic autoimmune liver disease that causes lymphocytic portal hepatitis and bile duct destruction, leading to cirrhosis and liver failure<sup>[1]</sup>. The annual incidence rates range between 0.7 and 49 cases per million persons, while prevalence rates range between 6.7 and 402 cases per million<sup>[2,3]</sup>. Currently, treatment with ursodeoxycholic acid (UDCA) at a dose of 13-15 mg/kg/d is the recommended first-line therapy.

According to clinical observations, the progression and prognosis of PBC vary significantly among patients, including a relatively slow progression in some patients, while others have a more rapid progression to hepatic failure<sup>[4,5]</sup>. Therefore, knowledge of the factors affecting disease progression and prognosis would be of great value in clinical management.

Several prognostic models have been developed for PBC. The most important is the Mayo score model, which has been widely used to predict the survival of PBC patients, and assess the timing of liver transplantation<sup>[6,7]</sup>. However, some patients are asymptomatic at the time of diagnosis, which limits the application of the Mayo model; and this model was not originally developed to assess disease progression in the early stages of PBC<sup>[8]</sup>. In terms of treatment, UDCA has been reported to delay histological progression and improve long-term prognosis<sup>[9]</sup>. Previous studies have shown that anti-gp210 and anti-centromere antibodies (ACA) were associated with severe disease course and poor prognosis. Bilirubin, prothrombin time, hypoalbuminemia and serum immunoglobulin (Ig) G have also been reported as prognostic markers<sup>[10-12]</sup>. However, these studies were mainly retrospective and had small sample sizes.

We aimed to assess the clinical features and risk factors for hepatic decompensation in a large prospective cohort study in China.

## MATERIALS AND METHODS

### Study population and data collection

Patients with PBC seen at the Peking Union Medical College Hospital (PUMCH) from 1995 to 2010 were recruited. The ethics committee at PUMCH approved the study, and written informed consent was obtained from all participants prior to enrollment. The diagnosis of PBC was made if at least two of the following three criteria were fulfilled: (1) elevated serum alkaline phosphatase (ALP) (at least 1.5 times the upper limit of normal); (2) the presence of anti-mitochondrial antibodies (AMAs) in serum; and (3) representative histological manifestations of portal area inflammation and bile duct injury. Early PBC was defined by seropositivity for AMA, and the presence of histological manifestations, but normal liver function<sup>[4]</sup>. The liver histology was graded according to the Ludwig classification<sup>[13]</sup>. Patients with liver decompensation, autoimmune hepatitis (AIH), viral hepatitis and other causes of liver damage were excluded. Patients

with PBC/AIH overlap syndrome were identified according to the criteria proposed by Chazouillères *et al*<sup>[14]</sup>. In the current study, serum antinuclear antibody (ANA), AMA, anti-smooth muscle antibody, anti-liver kidney microsomal antibody, and anti-parietal cell antibody were tested. In patients who were ANA positive, the level of alanine aminotransferase (ALT), aspartate aminotransferase (AST), IgG and autoantibodies for AIH were assessed to exclude PBC/AIH. The extra-hepatic autoimmune diseases were diagnosed according to the generally accepted criteria.

The following information was recorded: (1) demographic features, clinical signs and manifestations of liver disease including pruritus, persistent fatigue, jaundice and pain in the right hypochondrium; (2) laboratory parameters, including ANA, AMA, ACA, ALT, AST, ALP,  $\gamma$ -glutamyl-transferase (GGT), total bilirubin (TBil), direct bilirubin (DBil), albumin (ALB), IgG, IgA and IgM; and (3) imaging findings: liver ultrasound or CT demonstrating morphologic changes of liver and spleen, and portal vein blood flow. UDCA at the dose of 13-15 mg/kg per day was prescribed for all patients.

### Follow-up

Patients were followed up in the outpatient clinic, and data were collected at 3-mo intervals during the first year, 6-mo intervals or yearly thereafter. During each visit, the clinical signs, symptoms and laboratory parameters (AST, ALT, ALP, GGT, ALB, TBil, DBil, IgG, IgA, IgM, white blood cell, hemoglobin and platelet count) were assessed. Liver ultrasound was performed annually. Gastroscopy was carried out if necessary. If the patients had symptoms of hepatic decompensation (ascites, esophageal varices, variceal bleeding, hypersplenism, coagulant function abnormality, hypoproteinemia, and hepatic encephalopathy), they were permitted to visit doctors at any time or to go to emergency departments. After treatment for one year, patients whose ALP levels decreased by more than 40% of baseline values or returned to normal were defined as UDCA responders<sup>[15]</sup>.

### Outcome evaluation

Two clinical outcomes were studied: hepatic decompensation, and liver-related death. Hepatic decompensation was defined as the occurrence of severe functional damage of liver and one or more complications of liver cirrhosis<sup>[16]</sup>. We investigated the following features of decompensation: hypersplenism, ascites, esophageal varices (variceal bleeding), encephalopathy, hypoproteinemia, coagulant function abnormality, and spontaneous bacterial peritonitis. Figure 1 shows a diagram of the study design.

### Statistical analysis

Data were expressed as median (range) or mean  $\pm$  SD. Analysis was performed using SPSS 17.0 statistical software (Chicago, IL, United States). A two-sided *P*-value  $< 0.05$  was considered statistically significant. Categorical variables were compared by the  $\chi^2$  test while continuous

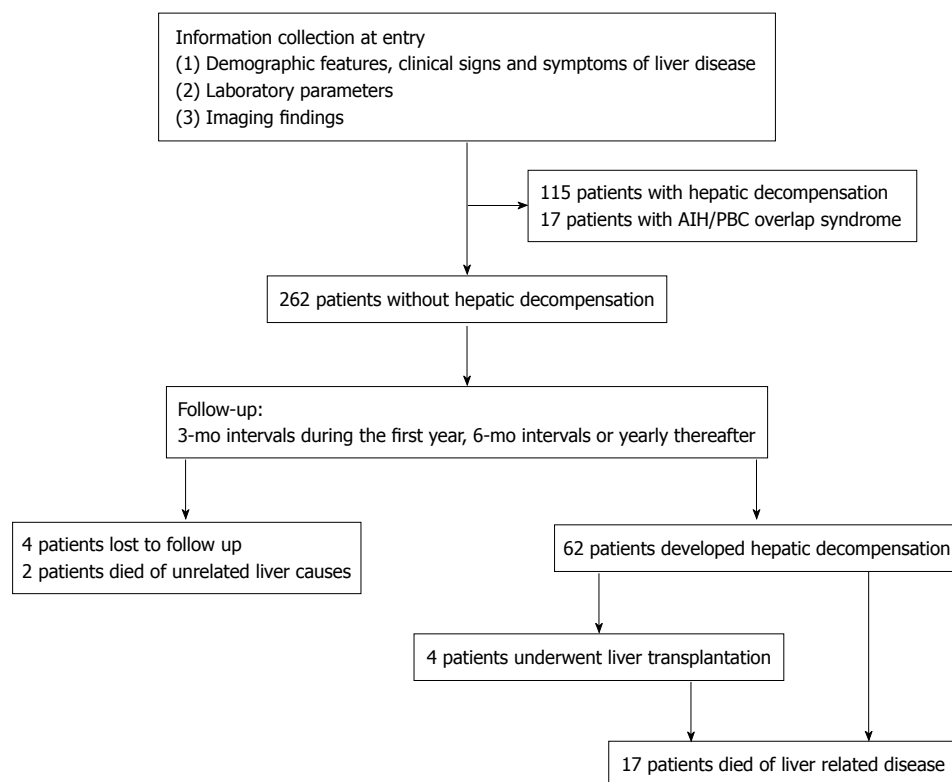


Figure 1 Diagram showing the study enrollment.

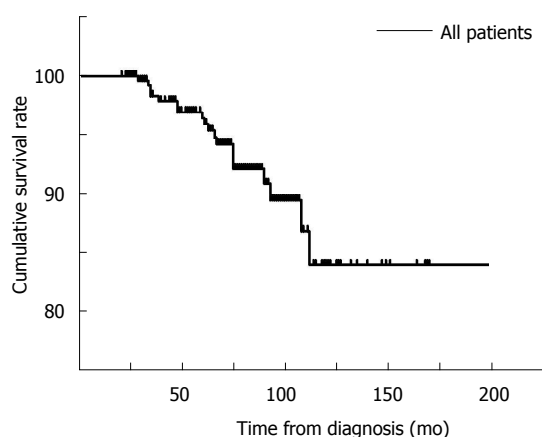


Figure 2 Survival curve of all patients in this cohort.

variables were compared by the Student's *t*-test or Mann-Whitney *U* test. Variables that were significant in univariate analysis were submitted to Cox regression analysis to predict the independent risk factors. Hazard risk (HR) and 95%CI were presented. Kaplan-Meier analysis was used to assess survival. Patients who were lost to follow-up or died of causes unrelated to liver failure were censored.

## RESULTS

### Clinical profiles of the study group at the entry

In this study, 262 patients with a mean age of  $51.5 \pm 10.2$  years, including 240 (91.6%) women, were enrolled.

The median duration of follow-up was 75.2 mo (range, 21-201 mo). The demographic and clinical features are shown in Tables 1 and 2.

On entry into the study, 170 (64.9%) patients presented with manifestations of liver disease, and 96 (36.6%) patients had extra-hepatic autoimmune disease. In addition, we enrolled 19 (7.3%) patients with early PBC. Two hundred and forty-five (93.5%) patients were seropositive for AMA. ANA and ACA were detected in 181 (69.1%) and 73 (27.9%) patients, respectively. Among 110 patients who underwent needle liver biopsies, 97 (88.2%) were at stages I-II, while 13 (11.8%) were at stages III-IV. One hundred and ninety (72.5%) patients received UDCA regularly and responded, while 46 patients (17.6%) took UDCA consistently, but failed to respond. Of the latter, eight patients terminated treatment on their own. Others (9.9%) did not take UDCA consistently, as prescribed.

### Incidence of outcomes and survival

During the study period, 62 (23.7%) patients developed adverse events, and the median duration from diagnosis to hepatic decompensation was 57.4 mo (range, 7-151 mo). Among these patients, four (1.5%) patients underwent liver transplantation at the end of follow-up or before death, and 17 (6.5%) patients died of liver failure. The median duration from diagnosis to death was 63.6 mo (range, 29-112 mo). According to Kaplan-Meier analysis, the cumulative survival rate and estimated median survival time were 83.9% and 181.7 mo, respectively (Figure 2 and Table 3).



**Table 1** Demographic and clinical laboratory characteristics of primary biliary cirrhosis patients at the time of entry ( $n = 262$ )  $n$  (%)

Age (yr), mean $\pm$ SD	51.5 $\pm$ 10.2
Gender (female/male), $n$	240/22
The symptomatic	170 (64.9)
Jaundice	78 (29.8)
Pruritus	69 (26.3)
Persistent fatigue	63 (24.0)
Pain in the right hypochondrium	40 (15.3)
UDCA responders	190 (72.5)
Early PBC	19 (7.3)
Extra-hepatic autoimmune diseases	96 (36.6)
Sjögren's syndrome	54 (20.6)
Autoimmune thyroid diseases	25 (9.5)
CREST/systemic sclerosis	8 (3.1)/3 (1.1)
Polymyositis/dermatomyositis	8 (3.1)/1 (0.4)
Systemic lupus erythematosus	4 (1.5)
Psoriasis	4 (1.5)
Rheumatoid arthritis	3 (1.1)
Takayasu's arteritis	2 (0.8)
Histological stages	110 (42.0)
I - II	97 (88.2)
III-IV	13 (11.8)

UDCA: Ursodeoxycholic acid; PBC: Primary biliary cirrhosis; PBC: Primary biliary cirrhosis; CREST: Comprehensive overview covers symptoms, causes, treatment of this autoimmune disorder.

### Risk factors for hepatic decompensation

Possible risk factors were first identified by univariate analysis. Subsequently, all variables, except histological stage, that showed statistical differences in univariate analysis were introduced into the Cox regression model. Incomplete UDCA response or inconsistent UDCA treatment ( $P < 0.001$ ; HR 95%CI = 2.423-7.541), ACA positivity ( $P < 0.001$ ; HR 95%CI = 2.516-7.137), alanine aminotransferase ratio (AAR) elevations ( $P < 0.001$ ; HR 95%CI = 1.357-2.678), TBil ( $P = 0.004$ ; HR 95%CI = 1.002-1.009), and ALP ( $P = 0.008$ ; HR 95%CI = 1.000-1.002) showed statistical significance in the model (Figure 3 and Tables 4 and 5). Although histological stage was not introduced into the model because of limited numbers, Cox regression analysis indicated that it was also a prognostic factor ( $P = 0.006$ ; HR 95%CI = 1.481-10.847; Figure 3).

## DISCUSSION

PBC is a chronic autoimmune liver disease that has the potential to progress to cirrhosis and, eventually, hepatic failure. Although it is less common in East Asia<sup>[17]</sup>, there is a rising frequency attributable to a more widespread awareness of this disease among physicians<sup>[18]</sup>. In this study, with a follow-up for 17 years, we identified the clinical profiles and risk factors for hepatic decompensation of PBC patients. To the best of our knowledge, this is the first prospective study with such a large number of patients in China.

In the current cohort, the demographic features and autoantibody profiles were consistent with those of previous studies<sup>[19]</sup>. Although PBC is an organ-specific dis-

**Table 2** Laboratory characteristics of primary biliary cirrhosis patients at the time of entry ( $n = 262$ )

ANA	181(69.1)
AMA	245 (93.5)
ACA	73 (27.9)
ALT (IU/L)	91.0 (9-598)
AST (IU/L)	86.2 (15-428)
AST/ALT	1.10 (0.22-4.85)
ALP (IU/L)	348.5 (21-1486)
GGT (IU/L)	428.2 (9-2614)
ALB (g/L)	40.7 (25-69)
TBil ( $\mu$ mol/L)	28.05 (4.90-334.40)
DBil ( $\mu$ mol/L)	14.39 (0.34-237.40)
IgG (g/L)	16.81 (5.12-56.60)
IgA (g/L)	3.15 (0.19-8.84)
IgM (g/L)	4.66 (0.41-22.30)

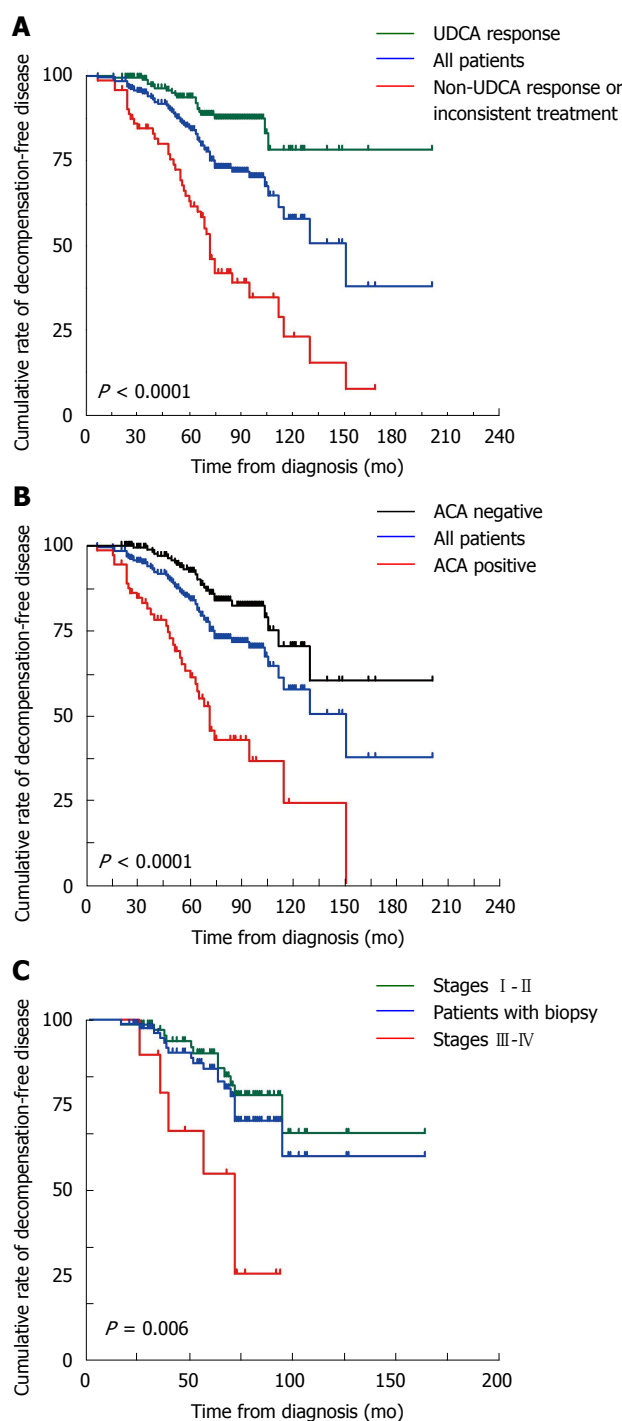
Continuous data were expressed as  $n$  (%) or median (range). ANA: Anti-nuclear antibody; AMA: Anti-mitochondrial antibody; ACA: Anti-centromere antibody; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT:  $\gamma$ -glutamyl-transferase; ALB: Albumin; TBil: Total bilirubin; DBil: Direct bilirubin; Ig: Immunoglobulin.

ease, association with extra-hepatic autoimmune diseases is an important characteristic. The prevalence of such co-existing diseases was high (36.6%), the most common of which were Sjögren's syndrome (SS) (20.6%) and autoimmune thyroid diseases (9.5%). These findings were similar to the results obtained by Marasini *et al*<sup>[20]</sup> in Italy and Silveira *et al*<sup>[21]</sup> in the United States.

Clinically, PBC patients can progress from an asymptomatic stage to a symptomatic stage because of liver damage<sup>[22]</sup>. In the current cohort, 64.9% patients had hepatic-related symptoms at diagnosis, which is higher than that reported by Prince *et al*<sup>[23]</sup>, but lower than the results by Su *et al*<sup>[11]</sup>. In the current study, the most common manifestation was jaundice, which was different from other reports in which the most common manifestation was fatigue<sup>[22,24,25]</sup>. The median survival time for asymptomatic patients has been reported to be longer than that for symptomatic patients<sup>[26]</sup>. In addition, other studies indicated that asymptomatic patients had shorter life spans than people in the general population<sup>[26,27]</sup>. However, several studies have indicated that asymptomatic patients did not have a better prognosis<sup>[23,28]</sup>.

In the current study, at the end of follow-up, sixty-two (23.7%) patients developed hepatic decompensation. Among them, all had portal hypertension, and 17 died of liver failure. Only four patients underwent transplantation because of the enormous financial cost for patients, and limited medical resources. However, the survival rate of our patients was similar to those presented in other recent studies<sup>[29-31]</sup>.

Although there has been controversy about efficacy, UDCA is currently accepted as the first-line drug for PBC. Several studies have shown that UDCA not only ameliorates laboratory indices, but also delays histological progression, and improves survival without transplantation<sup>[9,15,32-35]</sup>, especially for patients at early histological stages<sup>[33]</sup>. In the current study, 72.5% patients took



**Figure 3** Cumulative rates of decompensation-free disease in primary biliary cirrhosis patients stratified by ursodeoxycholic acid treatment, anti-centromere antibody, and histological stage, respectively. A: Ursodeoxycholic acid (UDCA); B: Anti-centromere antibody (ACA); C: Histological stage.

UDCA as prescribed and responded according to the Barcelona criteria; their prognoses were significantly better than those of non-responders, which is consistent with results of a previous study<sup>[9]</sup>. Therefore, the data suggest that all PBC patients should take UDCA regularly, but novel therapeutic options are needed for patients who do not respond to UDCA.

ACA had been observed in patients with various rheumatic disorders, including CREST syndrome (com-

**Table 3** Complications of chronic liver disease in the primary biliary cirrhosis cohort ( $n = 262$ )  $n$  (%)

Hepatic decompensation	62 (23.7)
Portal hypertension	62 (23.7)
Hypersplenism	44 (16.8)
Esophageal varices	47 (17.9)
Variceal bleeding	35 (13.4)
Ascites	43 (16.4)
Encephalopathy	19 (7.3)
Coagulant function abnormality	12 (4.6)
Hypoproteinemia	35 (13.4)
Spontaneous bacterial peritonitis	3 (1.1)
Liver transplantation	4 (1.5)
Death-liver related	17 (6.5) <sup>1</sup>
Censor	6 (2.3)
Lost to follow-up	4 (1.5)
Death from causes unrelated to liver failure	2 (0.8)

<sup>1</sup>One patient who underwent liver transplantation died.

prehensive overview covers symptoms, causes, treatment of this autoimmune disorder)<sup>[36]</sup>, SS<sup>[37]</sup>, Raynaud's phenomenon<sup>[38]</sup>, interstitial pneumonia in systemic lupus erythematosus<sup>[39]</sup>, SS overlap syndrome<sup>[40]</sup> and 15%-30% PBC patients<sup>[41-44]</sup>. In the current study, ACA was detected in 27.9% patients and 56.5% of decompensated patients. It was found to be an independent prognostic factor for patients. Previous cross-sectional studies have also indicated that ACA was associated with the development of liver failure<sup>[45]</sup> and progression to portal hypertension in PBC<sup>[10]</sup>. However, Rigamonti *et al*<sup>[46]</sup> found that PBC patients with or without ACA had the same rate of liver-related death, while Parveen *et al*<sup>[47]</sup> reported that ACA was not correlated with either laboratory or histological manifestations.

Elevation of AAR may be explained by a reduction in AST clearance, and by mitochondrial injury in severe liver disease<sup>[48]</sup>. A previous study showed that AAR exhibited modest correlations to Mayo and Child scores for evaluating the severity of PBC, and it was higher in histological stages III-IV than I-II disease. Patients with an AAR of 1 or less had better prognoses than their counterparts<sup>[49]</sup>. These results are consistent with the current data.

The beneficial value of liver biopsy in patients with PBC is controversial because the pattern of cirrhosis in PBC is irregular, and sampling error may lead to misinterpretation<sup>[50]</sup>. However, several studies have indicated a relationship between histology and clinical profile. AST and bilirubin were positively related to portal fibrosis for the AMA-positive patients<sup>[51]</sup>. Prognosis was found to be correlated with the histological stages of hepatic fibrosis, cholestasis and periportal cell necrosis<sup>[52]</sup>. Cirrhosis was found to have developed within four years in 31% and 50% of patients who were initially at stage I and stage II, respectively<sup>[53]</sup>. Cox analysis demonstrated that histological stage was a predictor of liver decompensation.

Although TBil and ALP were included in our model, the HR approached one. Therefore, they had a relatively low correlation with survival compared with other fac-

**Table 4** Univariate analysis of possible risk factors for hepatic decompensation in primary biliary cirrhosis

	Decompensation free ( <i>n</i> = 196)	Decompensation ( <i>n</i> = 62)	<i>P</i> value
Age at diagnosis (yr)	50.2	55.6	< 0.001
Gender (female/male)	178/18	59/3	0.275
Symptoms	60.20%	80.65%	0.003
UDCA treatment	85.20%	30.65%	< 0.001
ANA	64.80%	80.65%	0.019
AMA	92.86%	95.16%	0.731
ACA	19.39%	56.45%	< 0.001
ALT (IU/L)	89.6	96.6	0.109
AST (IU/L)	80.6	103.2	< 0.001
AST/ALT	1.05	1.27	0.013
ALP (IU/L)	311.6	454.1	< 0.001
GGT (IU/L)	395.4	524.3	0.001
ALB (g/L)	41.5	38.4	< 0.001
TBil (μmol/L)	20.92	48.84	< 0.001
DBil (μmol/L)	9.27	29.06	< 0.001
IgG (g/L)	16.61	17.43	0.386
IgA (g/L)	2.99	3.62	0.004
IgM (g/L)	4.61	4.83	0.285
Histological stage <sup>1</sup> (I - II)	92.22%	66.67%	0.008
Early PBC	9.70%	0.00%	0.023
Extra-hepatic autoimmune disease	37.24%	37.10%	0.983

<sup>1</sup>*n* = 108. ANA: Anti-nuclear antibody; AMA: Anti-mitochondrial antibody; ACA: Anti-centromere antibody; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: γ-glutamyl-transferase; ALB: Albumin; TBil: Total bilirubin; DBil: Direct bilirubin; UDCA: Ursodeoxycholic acid; Ig: Immunoglobulin.

tors, and their value for this purpose still needs to be confirmed by other, large long-term studies.

There are some limitations of the current study: (1) only patients without decompensation were enrolled, which resulted in the expected better survival rates compared to studies that included patients with decompensation; and (2) this was a single center study with a relatively short follow-up. Consequently, these risk factors still need to be confirmed further.

In summary, the clinical features and survival of patients with PBC in China are consistent with those described in Western countries. Incomplete UDCA response or inconsistent treatment, ACA positivity, higher AAR, and advanced histological stage are risk factors for hepatic decompensation.

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## COMMENTS

### Background

Primary biliary cirrhosis (PBC) is a chronic cholestatic autoimmune liver disease with a rising frequency, attributable to a widespread awareness from physicians. The progression and prognosis of PBC vary significantly among patients. However, the clinical features and risk factors for hepatic decompensation have not yet been well-documented in a large prospective cohort study in China.

**Table 5** Cox regression analysis of risk factors for hepatic decompensation in primary biliary cirrhosis (*n* = 262)

	HR	HR 95%CI	<i>P</i> value
Poor UDCA response or inconsistent treatment	4.275	2.423-7.541	< 0.001
ACA positivity	4.237	2.516-7.137	< 0.001
AAR	1.906	1.357-2.678	< 0.001
TBil	1.005	1.002-1.009	0.004
ALP	1.001	1.000-1.002	0.008

ACA: Anti-centromere antibody; ALP: Alkaline phosphatase; TBil: Total bilirubin; UDCA: Ursodeoxycholic acid; AAR: Alanine aminotransferase ratio; HR: Hazard risk.

## Research frontiers

In the current study, the authors identified the clinical profiles and risk factors for hepatic decompensation of PBC patients. In the authors' view, this is the first prospective study with such a large number of patients in China.

## Innovations and breakthroughs

Recent reports have highlighted the importance of ursodeoxycholic acid (UDCA) treatment for improving prognosis in patients with PBC. This is the first prospective cohort study showing that incomplete UDCA response or inconsistent treatment, anti-centromere antibodies positivity, higher alanine aminotransferase ratio, and advanced histological stage are risk factors for hepatic decompensation.

## Applications

Knowledge of the factors for hepatic decompensation in PBC patients would be of great value in clinical management. Novel treatments are needed for patients with poor prognostic factors.

## Terminology

PBC is an autoimmune liver disease characterized by the presence of anti-mitochondrial antibodies, and destruction of intrahepatic bile ducts, which can ultimately lead to cirrhosis and hepatic failure. Early UDCA treatment, excellent biochemical response to UDCA, and histological stages are predictors of survival.

## Peer review

The authors have documented the clinical profiles and analyzed risk factors for hepatic decompensation in patients with PBC. It is a relatively large prospective study and gives important information on the prognosis of PBC patients.

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## A temporary self-expanding metallic stent for malignant colorectal obstruction

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**RESULTS:** Stent placement and removal were technically successful in all patients with no procedure-related complications. Post-procedural complications included stent migration ( $n = 2$ ) and anal pain ( $n = 2$ ). Clinical success was achieved in 31 (93.9%) of 33 patients with resolution of bowel obstruction within 3 d of stent removal. Eleven of the 33 patients died  $73.81 \pm 23.66$  d (range 42-121 d) after removal of the stent without colonic re-obstruction. Clinical success was achieved in another 8 patients without symptoms of obstruction during the follow-up period. Reinsertion of the stent was performed in the remaining 12 patients with re-obstruction after  $84.33 \pm 51.80$  d of follow-up. The mean and median periods of relief of obstructive symptoms were  $97.25 \pm 9.56$  d and  $105 \pm 17.43$  d, respectively, using Kaplan-Meier analysis.

**CONCLUSION:** Temporary SEMS is a safe and effective approach in patients with malignant colorectal obstruction due to low complication rates and good medium-term outcomes.

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### Abstract

**AIM:** To investigate the clinical safety and efficacy of a temporary self-expanding metallic stent (SEMS) for malignant colorectal obstruction.

**METHODS:** From September 2007 to June 2012, 33 patients with malignant colorectal obstruction were treated with a temporary SEMS. The stent had a tubular configuration with a retrieval lasso attached inside the proximal end of the stent to facilitate its removal. The SEMS was removed one week after placement. Clinical examination, abdominal X-ray and a contrast study were prospectively performed and both initial and follow-up data before and at 1 d, 1 wk, and 1 mo, 3 mo, 6 mo and 12 mo after stent placement were obtained. Data collected on the technical and clinical success of the procedures, complications, need for re-insertion and survival were analyzed.

**Key words:** Self-expanding metallic stents; Colorectum; Malignant obstruction; Complications

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### INTRODUCTION

Over the last decade, colorectal stenting has been reported to be an effective method of relieving colonic obstruction in palliative treatment<sup>[1-5]</sup> or as a pre-operative bridge to facilitate one-stage surgical resection of primary colonic tumors<sup>[6-10]</sup>. Overall technical and clinical

success has been reported in 80%-100% of treated patients<sup>[11-15]</sup>. However, metallic stent placement has been plagued by tumor ingrowth (3%-46%) following bare stent placement<sup>[16-19]</sup>, perforation (4%)<sup>[12,13,15,20]</sup>, and stent migration (30%-50%)<sup>[10,21-24]</sup> following covered stent placement.

To overcome these problems, a temporary expandable colorectal stent was devised for use in patients with unresectable malignant colorectal obstruction. Our hypothesis was that the duration of placement of the self-expanding colorectal stent could be reduced to prevent perforation and stent migration. The purpose of this study was to evaluate the clinical safety and efficacy of a temporary self-expandable colorectal stent for the treatment of unresectable malignant colorectal obstruction, with a focus on preventing colonic perforation and stent migration.

## MATERIALS AND METHODS

### Study design

This pilot study was approved by the Institutional Review Board, and informed consent was obtained from all the patients. From September 2007 to June 2012, consecutive patients with malignant colorectal obstruction were treated with a temporary self-expanding metallic stent (Micro-Tech, Nanjing, China) in our department.

Diagnoses were established by reviewing patient histories, computed tomography (CT) imaging, colon studies and pathologic results. Patients were eligible for this procedure if the following criteria were met: (1) documented malignancy; (2) colorectal obstruction as defined by symptoms resulting in difficulty in defecation; (3) expandable metallic stent placement; and (4) life expectancy of more than 6 mo. Exclusion criteria were: (1) nonsymptomatic patients with malignant colorectal obstruction; (2) clinical evidence of perforation or peritonitis combined with multiple small-bowel obstructions; (3) extension of rectal cancer to the anal sphincter; and (4) right-sided acute obstruction (due to difficult access).

### Stent

The colon stent (Micro-Tech) is woven from a single thread of highly elastic nitinol wire 0.16 mm in diameter. The stent had a tubular configuration with an elliptic structure at the proximal and distal ends. The body section was 25-30 mm in diameter when fully expanded and 70-100 mm in length. The elliptic structure at the proximal and distal ends was 5 mm wider in diameter than the body section and 10 mm in length. To facilitate removal of the stent, a retrieval lasso was attached inside the proximal end of the stent to allow removal after placement. For implantation under fluoroscopic guidance, the stent was delivered in a compressed form inside an introducer sheath with a diameter of 16 F.

### Stent placement technique

The procedure was performed by one senior interventional radiologist (Yang RM), who had 18 years of ex-

perience in interventional radiology, under fluoroscopic guidance. Neither analgesia nor sedation was administered during the procedure.

Patients were initially placed in the left lateral decubitus position. Rotating the patient into the supine position allowed for a better anatomic view under fluoroscopy. The site of obstruction was established both endoscopically by direct vision and with water soluble contrast (Ultravist 300, Schering, Guangzhou, China) administered *via* a catheter passed through the endoscope and into the stricture. The procedure we used has been described elsewhere<sup>[1,6,7,10,25]</sup>. After the anal sphincter was lubricated, the distal ends of the tumors were identified by endoscopy. Then, a 0.035-inch guide wire (Radiofocus M; Terumo, Tokyo, Japan) with a 5-Fr catheter (Torcon NB; Cook, Bloomington, United States) was advanced and passed through the area of the stricture. The stricture location, length and morphology of the colon were identified by the injection of 30% diluted nonionic contrast medium (Ultravist) *via* the catheter. To avoid perforation, balloon dilation was not generally performed. After exchanging the guide wire for a 0.035-inch super-stiff guide wire (Boston Scientific/Medi-Tech, Watertown, United States), a 16-Fr delivery system (Micro-Tech) was passed over the stiff guide wire until the proximal and distal edges of the prosthesis bridged the stricture under fluoroscopic control, and the stent was then deployed by pulling back the introducer sheath. Finally, contrast medium (Ultravist) was injected through the catheter to assess correct placement and expansion of the stent.

Upon completion of the procedure, patients were transferred to the ward for observation. Once obstructive symptoms had remitted about one week after stent placement, the stent was grasped by the retrieval lasso and gently pulled out. A contrast study was performed to evaluate the patency to rule out possible concomitant lesions in the proximal and distal colon.

### Postoperative outcome evaluation

Before SEMS placement, a routine workup, including CT of the abdomen and chest, as well as calculation of Karnofsky performance status, was conducted. An abdominal radiograph was taken during hospitalization 1-3 d after placement to confirm the correct deployment and expansion. After successful removal of the stent, patients were monitored in the outpatient clinic until either death, surgery was performed, patient was lost to follow-up or a complication developed. Clinical examination, abdominal X-ray and a contrast study were performed by two of our authors, who gathered both initial and follow-up data before and at 1 d, 1 wk, and 1 mo, 3 mo, 6 mo and 12 mo after stent placement. In cases where clinical exams could not be performed, data were obtained by means of telephone calls to the patient or the closest relative and by reviewing medical records. CT examination or an endoscopic procedure was performed if there was persistence or reappearance of symptoms such as abdominal pain, constipation, or rectal bleeding. Technical and clinical success of the procedure, occur-

rence and timing of complications, and the need for surgical intervention were analyzed.

Patients were considered to have incurable cancer when curative resection of metastatic disease was impossible due to extensive liver metastases or extrahepatic disease. Technical success was defined as successful placement of the SEMS, with correct deployment, positioning at the level of the stenosis, and removal of the stent determined with radiologic procedures. Clinical success was defined as complete colonic decompression and relief of obstructive symptoms as judged by clinical symptoms and radiographic observations, without intervention or device-related complications within 72 h after SEMS removal. Death was considered to be related to SEMS complications if the patient died within 7 d of insertion or removal. Major complications were events leading to surgery or reintervention or requiring admission to the intensive care unit. Perforation, stent obstruction, and migration were considered to be major complications. Mild complications were events leading to rehospitalization or prolonged hospital stay without fulfilling the major complications criteria.

### Statistical analysis

Descriptive data are expressed as the mean  $\pm$  SD. Categorical data are reported as numbers and percentages. Time to considered end points (occurrence of complications, surgical intervention, or death) was determined. Kaplan-Meier analysis of relief of obstructive symptoms was performed to calculate the cumulative rate of clinical success, such as sustained relief of obstruction and lack of complications. All statistical analyses were performed using the SPSS package, version 13.0 (SPSS, Chicago, Illinois, United States).

## RESULTS

### Patients

A total of 38 patients were included in this study initially. Five of these were excluded due to patients being lost to follow-up, thus, a total of 33 eligible patients were included (19 men, 14 women; mean age  $61.55 \pm 14.59$  years, range 30–85 years). Stenoses were located in: the transverse colon ( $n = 3$ ); left colon ( $n = 7$ ); sigmoid colon ( $n = 12$ ); rectum ( $n = 11$ ). The mean distance of the lesion from the anus was 19.23 cm (range 5–71 cm), and the mean lesion length was  $5.97 \pm 2.07$  cm (range 4–12 cm). The obstructions were complete (with no passage of contrast medium during contrast studies before or during stent placement) in 8 patients and incomplete in the remaining 25 patients.

### Technical and initial clinical results

Stent placement in the target colon stricture was technically successful in all patients without procedure-related complications. Initially, all patients required the placement of one stent to cover the length of the obstruction. Complete expansion of the placed stent occurred within 2 d of stent placement. No patient underwent balloon

dilation, either before or after stent placement. The mean procedure time was 42 min (range 30–120 min). Removal of the stents was successful in all patients.

In patients with technically successful removal of the stent, clinical success was achieved in 31 of the 33 patients within 72 h of removal, with a success rate of 93.9%. Two patients who did not achieve clinical success after removal of the stents due to paralytic ileus or extension of the tumor were retreated with the stent.

### Complications

No perforations occurred following placement or removal of the stents. Stent migration occurred in 2 patients (distal partial migration in two patients), and none of these patients required a second stent placement due to improvement of the obstruction at the time of stent removal. Two patients who had a stent placed in the rectum complained of moderate rectal pain within 2 d of stent placement without requiring analgesics.

### Follow-up results

Eleven of the 33 patients died  $73.81 \pm 23.66$  d (range 42–121 d) after removal of the stent without colonic re-obstruction due to diffuse metastatic cancer, cachexia, or myocardial infarction. Clinical success was achieved in 8 patients without symptoms of obstruction. Stent reinsertion was performed in the remaining 12 patients with re-obstruction after a mean follow-up period of  $84.33 \pm 51.80$  d (range 27–201 d). The mean and median periods of relief of obstructive symptoms were  $97.25 \pm 9.56$  d (95%CI: 79, 116) and  $105 \pm 17.43$  d (95%CI: 70, 139), respectively, using Kaplan-Meier analysis.

## DISCUSSION

The present study was designed to test the hypothesis that a temporary self-expanding metallic colorectal stent could reduce the risk of colonic perforation and stent migration. In this uncontrolled prospective study of 33 patients with unresectable malignant colorectal obstruction, temporary self-expanding metallic colorectal stent placement was technically successful in all patients with a clinical success rate of 93.9%. There was no procedure-related mortality or perforations in this study, and all complications were managed without surgical intervention. These results suggest that a temporary self-expanding metallic colorectal stent can be considered a viable and effective treatment for patients with unresectable malignant colorectal obstruction. To date, this is the first report to describe the treatment of unresectable malignant colorectal obstruction with a temporary self-expanding metallic colorectal stent.

Colonic or rectal stent placement is associated with some complications, including stent migration, perforations, rectal bleeding, fecal impaction, abdominal pain, and tenesmus, of which stent migration and perforation are the most serious complications. In systematic reviews, migration was reported to occur in approximately 10%–12% of patients<sup>[15,20]</sup>, and is usually detected on



follow-up radiographs within 1 wk of insertion. A comparatively small diameter and limited flexibility may have contributed to the more frequent occurrence of stent migration in earlier studies<sup>[26]</sup>. Although, Repici *et al*<sup>[1]</sup> and Song *et al*<sup>[6]</sup> have reported a lower migration rate (2% and 3%, respectively) with the use of the newly designed colorectal stents - WallFlex stent and dual stent (with a large diameter and flared ends) in the treatment of malignant colorectal obstruction, however, these stents may result in another serious complication - perforation<sup>[6]</sup>. Song *et al*<sup>[6]</sup> reported the lowest migration rate (0%) in the bridge-to-surgery group, but they also yielded the highest complication rate - colon perforation (22%) in 11 of the 50 patients in the same group after successful placement of the dual stent.

Perforation is found in 3.7%-4.0% of patients with a colorectal SEMS<sup>[15,20]</sup>, which is lower than stent migration, but it is the most serious complication which may threaten the life of patients. Balloon dilatation of a stricture to obtain access can result in excessive manipulation of the wire through the colonic wall and anatomical sites with a comparatively high perforation risk. It is probable that stent design and long-term placement may play an important role in colonic perforation. Large diameter stents with flared ends and the stent eroding through the colonic wall during colonic peristalsis may directly result in perforation.

The ideal colorectal stent should have adequate radial expansile force and smooth edges. Enough radial expansile force is spontaneously and evenly generated, and the final diameter is reached over the course of 2-5 d, so that dilation of the stricture is gentle, as well as effective<sup>[27]</sup>. Thus, predilation is not generally necessary, and the potential for migration is reduced. A colorectal stent with smooth edges, obviating sharp hooks in the stent design, can maximally reduce the risk of perforation. The stent made by Micro-Tech has a relatively large profile and an elliptic structure to prevent stent migration, and the elliptic structure has a shrunk edge at both sides to minimize the risk of perforation and satisfies the requirements for prevention of perforation.

The significant improvement in perforation rate and stent migration during the follow-up period in the present study was predominantly attributable to the stent design and its temporary use, and demonstrated that the stent can be safely used in malignant colorectal obstruction. Our 93.9% clinical success rate in the relief of colonic obstruction following stent removal was in line with that of other researchers who reported 80%-100% relief of colonic obstruction in patients with malignant colorectal obstruction treated with SEMS<sup>[5,9,14,15,20]</sup>, and demonstrated the efficacy of the temporary stent for patients with malignant colorectal obstruction.

Our results have important clinical implications. The use of a temporary self-expanding metallic colorectal stent rather than a long-term self-expanding metallic colorectal stent in patients with unresectable malignant colorectal obstruction will substantially decrease the risk of perforation and stent migration. The findings from

this study may encourage more studies on temporary self-expanding metallic colorectal stents in the palliative treatment of patients with unresectable malignant colorectal obstruction.

Our study has some limitations. The number of patients treated was relatively small with a short lifespan, and death due to rapid progression of the disease may have masked both the benefits and risks of the procedure. Secondly, we did not include a control group, therefore, future randomized trials are needed to compare the temporary self-expanding metallic colorectal stent with the long-term self-expanding metallic colorectal stent in terms of efficacy, risk of complications, and recurrent obstruction, with particular attention to stent migration, tumoral and nontumoral tissue overgrowth and perforation.

In conclusion, our preliminary study demonstrated that the temporary SEMS was a safe and effective approach for colon decompression in patients with colorectal malignant obstruction, with a low rate of complications and good medium-term outcomes. Reinsertion of the stent can be performed in patients with re-obstruction. Although the initial results are promising, longer follow-up and expanded clinical trials are needed.

## COMMENTS

### Background

Colorectal stenting has been reported to be an effective method of relieving colonic obstruction, however, metallic stent placement has been plagued by tumor ingrowth, perforation and migration.

### Research frontiers

A temporary self-expanding metallic stent (SEMS) has been widely used for the treatment of patients with malignant esophageal stenosis, however, the use of this type of stent in patients with malignant colorectal obstruction has rarely been reported. Authors investigated the clinical safety and efficacy of a temporary self-expandable colorectal stent for malignant colorectal obstruction.

### Innovations and breakthroughs

A temporary SEMS was specially devised for the management of patients with malignant colorectal obstruction associated with colonic cancer. All procedures were performed under fluoroscopic control. This is the first study to report the use of a temporary SEMS for malignant colorectal obstruction.

### Applications

By determining the efficacy and safety of a temporary SEMS, this may provide a new therapeutic intervention for the treatment of patients with malignant colorectal obstruction.

### Terminology

Malignant colorectal obstruction is a common presentation of colorectal cancer, accounting for about 15%-20% of initial presentations in patients with colorectal malignancy.

### Peer review

The authors present a prospective study of temporary SEMS for malignant colorectal obstruction, with the aim of investigating the clinical safety and efficacy of the approach. The results reveal that the clinical success was achieved in 31 (93.9%) of 33 patients with resolution of bowel obstruction within 3 d after stent removal, as well as lower complication rates. In addition, the mean and median periods of relief of obstructive symptom were  $97.25 \pm 9.56$  d and  $105 \pm 17.43$  d, respectively. The results are interesting. A temporary SEMS may be a good attempt to seek the new clinical therapy for unresectable colorectal cancer patients and has a potential clinical significance.

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## Roux-en-Y versus Billroth I reconstruction after distal gastrectomy for gastric cancer: A meta-analysis

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Operative outcomes such as operation time, intraoperative blood loss and postoperative outcomes such as anastomotic leakage and stricture, bile reflux, remnant gastritis, reflux esophagitis, dumping symptoms, delayed gastric emptying and hospital stay were the main outcomes assessed. Meta-analyses were performed using RevMan 5.0 software (Cochrane library).

**RESULTS:** Four randomized controlled trials (RCTs) and 9 non-randomized observational clinical studies (OCS) involving 478 and 1402 patients respectively were included. Meta-analysis of RCTs revealed that R-Y reconstruction was associated with a reduced bile reflux (OR 0.04, 95%CI: 0.01, 0.14;  $P < 0.0001$ ) and remnant gastritis (OR 0.43, 95%CI: 0.28, 0.66;  $P = 0.0001$ ), however needing a longer operation time (WMD 40.02, 95%CI: 13.93, 66.11;  $P = 0.003$ ). Meta-analysis of OCS also revealed R-Y reconstruction had a lower incidence of bile reflux (OR 0.21, 95%CI: 0.08, 0.54;  $P = 0.001$ ), remnant gastritis (OR 0.18, 95%CI: 0.11, 0.29;  $P < 0.0001$ ) and reflux esophagitis (OR 0.48, 95%CI: 0.26, 0.89;  $P = 0.02$ ). However, this reconstruction method was found to be associated with a longer operation time (WMD 31.30, 95%CI: 12.99, 49.60;  $P = 0.0008$ ).

**CONCLUSION:** This systematic review point towards some clinical advantages that are rendered by R-Y compared to B-I reconstruction post DG. However there is a need for further adequately powered, well-designed RCTs comparing the same.

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**Key words:** Gastric cancer; Distal gastrectomy; Roux-en-Y; Billroth I; Reconstruction; Meta-analysis

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### Abstract

**AIM:** To conduct a meta-analysis to compare Roux-en-Y (R-Y) gastrojejunostomy with gastroduodenal Billroth I (B-I) anastomosis after distal gastrectomy (DG) for gastric cancer.

**METHODS:** A literature search was performed to identify studies comparing R-Y with B-I after DG for gastric cancer from January 1990 to November 2012 in Medline, Embase, Science Citation Index Expanded and the Cochrane Central Register of Controlled Trials in The Cochrane Library. Pooled odds ratios (OR) or weighted mean differences (WMD) with 95%CI were calculated using either fixed or random effects model.



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## INTRODUCTION

Gastric cancer is one of the most common cancers worldwide with approximately 989 600 new cases and 738 000 deaths per year, accounting for about 8 percent of new cancers<sup>[1]</sup>. Surgical resection is still the only option for providing definitive treatment of this malignant disease<sup>[2]</sup>. Due to the early diagnosis of gastric cancer, there has been a significant improvement in the long-term survival of patients undergoing surgery within the past decade<sup>[3]</sup>. Surgeons have therefore focused on improving the patients' postoperative quality of life by modifying the surgical technique and the type of reconstruction performed after distal gastrectomy (DG)<sup>[4]</sup>. The three mainly used reconstruction techniques after DG are: (1) gastroduodenal anastomosis (Billroth- I, B- I); (2) gastrojejunal anastomosis (Billroth- II, B- II); and (3) Roux-en-Y (R-Y) gastrojejunostomy. Although both B- I and R-Y anastomoses are recognized as standard reconstruction procedures after DG<sup>[5]</sup>, it is yet to be established, which of these is the better of the two. The B- I reconstruction has been commonly performed, because of its technical simplicity, with only one anastomotic site and maintaining physiological intestinal continuity<sup>[5,6]</sup>. However, gastroesophageal and duodenogastric reflux are well documented in patients who undergo this type of reconstruction following DG<sup>[7]</sup>, and severe gastritis, esophagitis and gastric cancer can subsequently occur<sup>[8-11]</sup>. The aforementioned complications seriously affect postoperative quality of life of patients undergoing DG<sup>[12]</sup>.

For several decades, R-Y reconstruction has been the preferred method to prevent reflux gastritis, esophagitis and decrease probability of gastric cancer recurrence<sup>[9,10,13,14]</sup>. However, the choice of surgical reconstruction is often based on personal preferences of surgeons, *e.g.*, majority of surgeons in the East favor a B- I reconstruction, while R-Y is the procedure of choice in the West<sup>[9,15]</sup>. Although a few studies have directly compared B- I and R-Y techniques, these studies have failed to reach a consensus and establish which method is the best choice after DG for gastric carcinoma. Thus it is difficult to choose a particular type of reconstruction, based on the current evidence base. We therefore sought to compare the perioperative outcomes and postoperative complications of patients undergoing R-Y and B- I reconstruction after DG for gastric cancer by undertaking a meta-analysis of published data.

## MATERIALS AND METHODS

### Literature search

A comprehensive literature search of Medline, Embase, Science Citation Index Expanded and the Cochrane Cen-

tral Register of Controlled Trials in the Cochrane Library between January 1990 and November 2012 was carried out for comparing R-Y and B- I reconstructions after DG for gastric cancer. Medical subject headings as well as keywords "Roux-en-Y"; "Billroth- I"; "reconstruction"; "distal gastrectomy"; "gastric cancer" and "stomach cancer" were used. All abstract supplements from published literature were searched manually. Relevant papers were also identified from the reference lists of previous papers, including those obtained through the search of abstracts and recent international meetings. Randomized controlled trials (RCTs) and non-randomized observational clinical studies (OCS) with full-text descriptions were included. Final inclusion of articles was determined by consensus; when this failed, a third author adjudicated. The results of the search strategy are shown in Figure 1.

### Inclusion criteria and exclusion criteria

Two authors identified and screened the search findings for potentially eligible studies. Inclusion criteria were: (1) English language articles published in peer-reviewed journals; (2) Human trials of patients with gastric cancer undergoing DG as the main procedure; (3) Studies with at least one of the outcomes mentioned; and (4) When similar studies were reported by the same institution or author, either the better quality study or the more recent publication was included. Following studies were excluded: (1) Abstracts, letters, editorials, expert opinions, reviews and case reports; (2) Studies without available data; (3) Studies without control group; and (4) Studies including patients with benign disease.

### Outcomes of interest

Perioperative outcomes and postoperative complications were evaluated. Operation time, intraoperative blood loss and hospital stay were the main perioperative outcomes to be assessed. Postoperative complications included anastomotic leakage and stricture, bile reflux, remnant gastritis, reflux esophagitis, dumping symptoms and delayed gastric emptying.

### Data extraction and quality assessment

Data were extracted by two independent observers using standardized forms. RCTs were qualitatively analyzed using Jadad scoring system<sup>[16]</sup>. Non-randomized OCS were similarly evaluated using Newcastle-Ottawa scoring system<sup>[17]</sup>. The quality assessment was also carried out by two independent observers and is displayed in Tables 1 and 2. Quantitative data extracted from the selected studies including: population characteristics (study year, country, design, gender, mean age) and outcome parameters (operation time, intraoperative blood loss, hospital stay, anastomotic leakage and stricture, bile reflux, remnant gastritis, reflux esophagitis, dumping symptoms and delayed gastric emptying).

### Statistical analysis

Meta-analyses were performed by using Review Man-



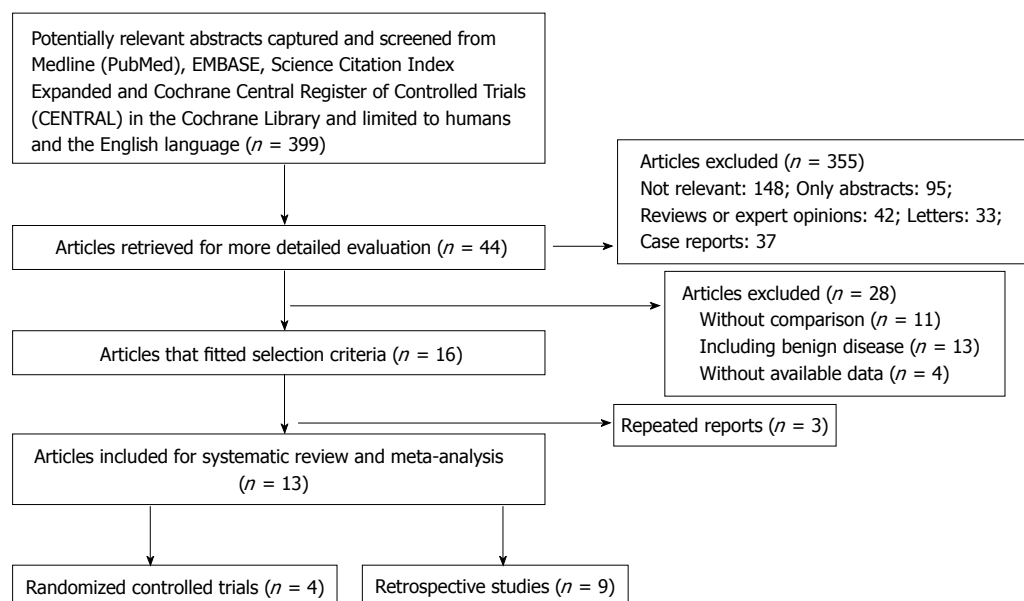


Figure 1 Flow diagram depicting the process of identification and inclusion of selected studies.

Table 1 Quality assessment of the included randomized controlled studies based on the Jadad scoring system

Ref.	Randomized	Appropriate randomization	Appropriately double blinded	Description of withdrawals	Total Jadad score
Ishikawa <i>et al</i> <sup>[20]</sup>	Yes	Yes	No	Yes	3
Lee <i>et al</i> <sup>[26]</sup>	Yes	Yes	No	Yes	3
Imamura <i>et al</i> <sup>[27]</sup> or Hirao <i>et al</i> <sup>[31]</sup>	Yes	Yes	No	Yes	3

Table 2 Newcastle-Ottawa scoring system for nonrandomized comparative studies

Ref.	Selection star	Comparability star <sup>1</sup>	Outcome star	Total star
Osugi <i>et al</i> <sup>[9]</sup>	3	2	3	8
Shinoto <i>et al</i> <sup>[10]</sup>	3	2	3	8
Nunobe <i>et al</i> <sup>[13]</sup>	4	2	3	9
Fukuhara <i>et al</i> <sup>[14]</sup>	3	2	3	8
Kojima <i>et al</i> <sup>[21]</sup>	3	2	3	8
Namikawa <i>et al</i> <sup>[22]</sup>	3	2	2	7
Tanaka <i>et al</i> <sup>[23]</sup>	3	2	3	8
Kumagai <i>et al</i> <sup>[24]</sup>	3	2	1	6
Kim <i>et al</i> <sup>[25]</sup>	3	2	2	7

<sup>1</sup>Factors considered: Age, gender; American Society of Anesthesiologists grading and tumor stage.

ager Version 5.0 software (The Cochrane Collaboration, Oxford, United Kingdom). For categorical variables, treatment effects were expressed as odds ratio (OR) with corresponding 95% CIs. For continuous variables, treatment effects were expressed as weighted mean difference (WMD) with corresponding 95% CI. Meta-analyses were performed using fixed- or random-effects model, depending on the absence or presence of significant heterogeneity. Heterogeneity was evaluated using the  $\chi^2$  test, with a  $P < 0.1$  was considered significant;  $I^2$  values were used for the evaluation of statistical heterogeneity<sup>[18]</sup>. If the test rejected the assumption of homogeneity of stud-

ies, then the random effects analysis was performed<sup>[19]</sup>. Sensitivity analyses were also performed by removing individual studies from the data set and analyzing the effect on the overall results to identify sources of significant heterogeneity. Funnel plots were constructed to evaluate potential publication bias.

## RESULTS

### Study characteristics

There were 399 papers relevant to the search terms. Sixteen studies<sup>[9,10,13,14,20-31]</sup> met the inclusion criteria. Four studies had previously been reported by the same institution<sup>[27-29,31]</sup>. Three studies were excluded<sup>[27-29]</sup>, however, one study had some outcomes which we can include<sup>[31]</sup>. Finally, four RCTs<sup>[20,26,27,31]</sup>, and 9 OCS<sup>[9,10,13,14,21-25]</sup> with 478 and 1402 patients respectively were included. All these studies have been carried out in Japan and Korea. The number of patients in the included studies ranged from 43 to 424. Characteristics of studies included in the meta-analysis are presented in Table 3.

### Meta-analysis results

Included RCTs and OCS were analyzed separately to determine outcome measures in the study groups. All the results are summarized in Table 4.

**RCTs comparison:** To date, 4 RCTs have been under-

**Table 3** Characteristics of included studies in the meta-analysis

Ref.	Country	Design	Group	Patients (n)	Male/female (n)	Mean age (yr)
Osugi <i>et al</i> <sup>[9]</sup>	Japan	Retro	R-Y	18	13/5	60.2
Shinoto <i>et al</i> <sup>[10]</sup>	Japan	Retro	B- I	25	12/13	64.7
			R-Y	20	NR	63 ± 12
Nunobe <i>et al</i> <sup>[13]</sup>	Japan	Retro	B- I	43	NR	63 ± 9
			R-Y	182	117/65	58.8
Fukuhara <i>et al</i> <sup>[14]</sup>	Japan	Retro	B- I	203	127/76	58.7
			R-Y	29	23/6	56.1
Ishikawa <i>et al</i> <sup>[20]</sup>	Japan	RCT	B- I	41	19/22	66.0
			R-Y	24	17/7	64 (43-80)
Kojima <i>et al</i> <sup>[21]</sup>	Japan	Retro	B- I	26	19/7	61 (34-84)
			R-Y	68	43/25	62.8 ± 12.2
Namikawa <i>et al</i> <sup>[22]</sup>	Japan	Retro	B- I	65	48/17	62.0 ± 8.9
			R-Y	38	22/16	71 (41-80)
Tanaka <i>et al</i> <sup>[23]</sup>	Japan	Retro	B- I	47	25/22	72 (33-86)
			R-Y	51	34/17	65.2
Kumagai <i>et al</i> <sup>[24]</sup>	Japan	Retro	B- I	50	34/16	66.2
			R-Y	95	74/21	62.7 (42-81)
Kim <i>et al</i> <sup>[25]</sup>	South Korea	Retro	B- I	329	197/132	63.5 (29-90)
			R-Y	26	21/5	≥ 60 (9)
Lee <i>et al</i> <sup>[26]</sup>	South Korea	RCT	B- I	72	54/18	≥ 60 (41)
			R-Y	47	28/19	58.5 ± 10.7
Imamura <i>et al</i> <sup>[27]</sup> or Hirao <i>et al</i> <sup>[31]</sup>	Japan	RCT	B- I	49	31/18	60.0 ± 11.6
			R-Y	169	115/54	63.9 ± 10.5
			B- I	163	105/58	64.4 ± 9.3

Retro: Retrospective observational study; RCT: Randomized controlled trial; R-Y: Roux-en-Y; B- I : Billroth- I .

**Table 4** Meta-analysis of outcomes of interest

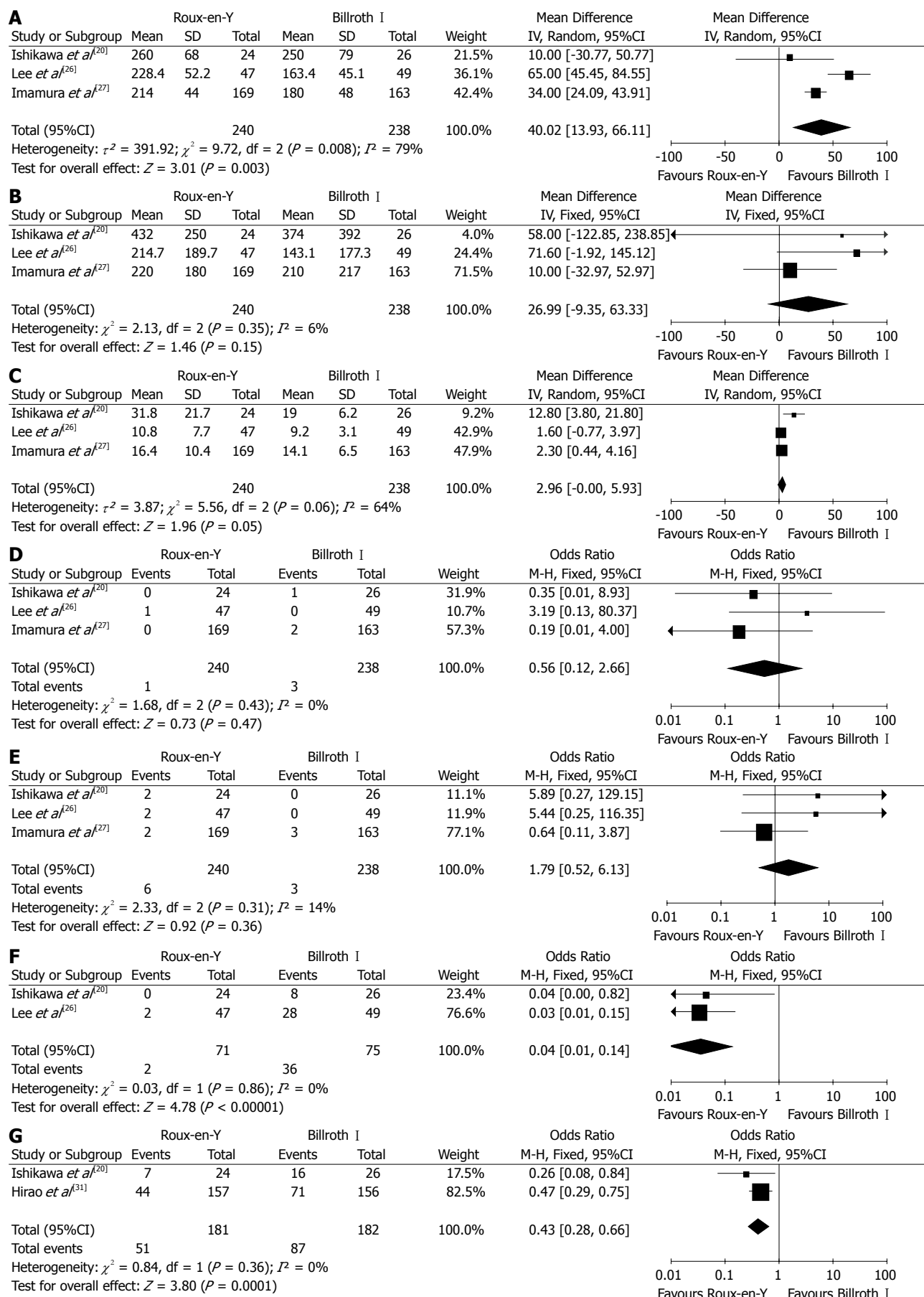
Outcome of interest	Studies	Patients (n)	OR/WMD	95%CI	P value
RCT					
Operation time	3	478	40.02	13.93, 66.11	0.003
Intraoperative blood loss	3	478	26.99	-9.35, 63.33	0.15
Hospital stay	3	478	2.96	-0.00, 5.93	0.05
Anastomotic leakage	3	478	0.56	0.12, 2.66	0.47
Anastomotic stricture	3	478	1.79	0.52, 6.13	0.36
Bile reflux	2	145	0.04	0.01, 0.14	< 0.00 001
Reflux esophagitis	3	458	0.49	0.20, 1.23	0.13
Remnant gastritis	2	363	0.43	0.28, 0.66	0.000
Delayed gastric emptying	2	363	2.31	0.12	44.41
OCS					
Operation time	4	718	31.3	12.99, 49.60	0.001
Intraoperative blood loss	3	620	26.9	-46.54, 100.34	0.47
Hospital stay	2	522	1.40	-0.17, 2.97	0.08
Anastomotic leakage	5	813	1.26	0.40, 3.97	0.70
Anastomotic stricture	3	658	0.94	0.31, 2.89	0.91
Bile reflux	6	757	0.21	0.08, 0.54	0.001
Reflux esophagitis	5	719	0.48	0.26, 0.89	0.02
Remnant gastritis	6	784	0.18	0.11, 0.29	< 0.00 001
Dumping symptoms	4	347	0.59	0.32, 1.12	0.11
Delayed gastric emptying	4	701	0.95	0.24, 3.74	0.94

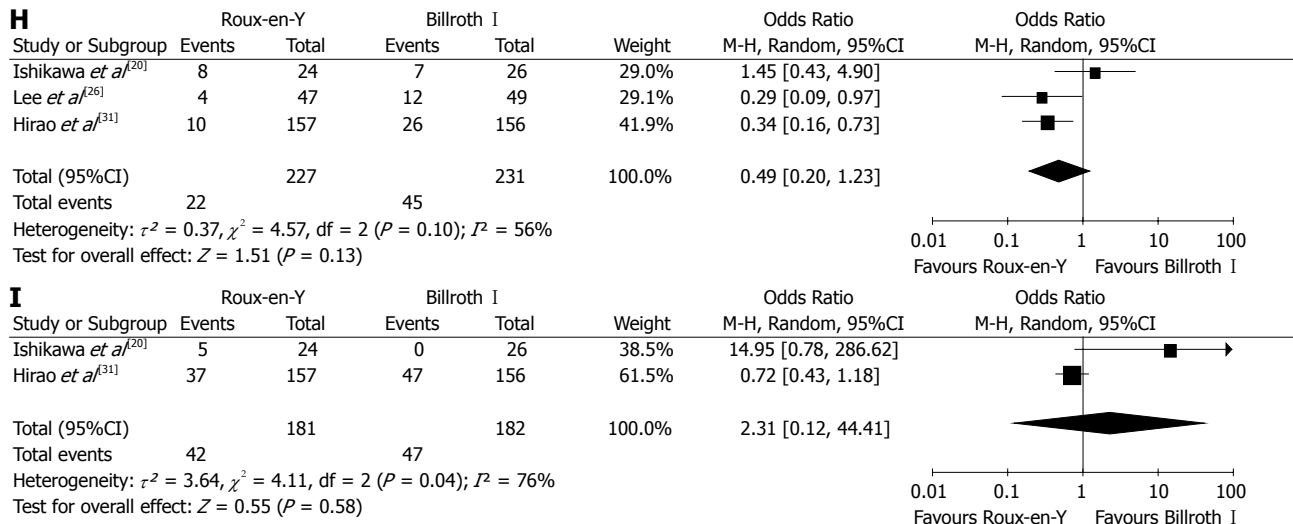
RCT: Randomized controlled trials; OCS: Non-randomized observational clinical studies; OR: Odds ratio; WMD: Weighted mean differences.

taken to compare R-Y with B- I reconstruction<sup>[20,26,27,31]</sup>. However, two studies<sup>[27,31]</sup> have same study populations. All studies had a clear description of the sample size calculation and were found to be of high quality according to Jadad scoring system. The detailed results of meta-analysis are given in Figure 2.

Meta-analysis revealed that R-Y reconstruction was associated with a significant reduction in the incidence

of bile reflux (OR 0.04, 95%CI: 0.01, 0.14;  $P < 0.00001$ ) and remnant gastritis (OR 0.43, 95%CI: 0.28, 0.66;  $P = 0.0001$ ). No significant differences were observed between the groups in terms of intraoperative blood loss (OR 26.99, 95%CI: -9.35, 63.33;  $P = 0.15$ ), hospital stay (OR 2.96, 95%CI: -0.00, 5.93;  $P = 0.05$ ), anastomotic leakage (OR 0.56, 95%CI: 0.12, 2.66;  $P = 0.47$ ), stricture (OR 1.79, 95%CI: 0.52, 6.13;  $P = 0.92$ ), reflux esopha-





**Figure 2 Roux-en-Y versus Billroth I -randomized controlled trials comparison.** A: Operation time; B: Intraoperative blood loss; C: Hospital stay; D: Anastomotic leakage; E: Anastomotic stricture; F: Bile reflux; G: Remnant gastritis; H: Reflux esophagitis; I: Delayed gastric emptying. Pooled weighted mean difference (WMD) or odds ratio (OR) with 95%CI was calculated using the fixed-or random effects model.

gitis (OR 0.49, 95%CI: 0.20, 1.23;  $P = 0.13$ ) and delayed gastric emptying (OR 2.31, 95%CI: 0.12, 44.41;  $P = 0.58$ ). However, B- I reconstruction method took significantly less time to perform as compared to R-Y reconstruction (WMD 40.02, 95%CI: 13.93, 66.11;  $P = 0.003$ ).

Only one study<sup>[20]</sup> reported incidence of dumping symptoms. The incidence of dumping symptoms was not significantly different between the two groups (OR 1.09, 95%CI: 0.14, 8.42;  $P = 0.93$ ).

**OCS comparison:** Nine OCS were included<sup>[9,10,13,14,21-25]</sup>. Forest plots are illustrated in Figure 3. Results suggested that R-Y reconstruction had significantly lower incidence of bile reflux (OR 0.21, 95%CI: 0.08, 0.54;  $P = 0.001$ ), remnant gastritis (OR 0.18, 95%CI: 0.11, 0.29;  $P < 0.0001$ ) and reflux esophagitis (OR 0.48, 95%CI: 0.26, 0.89;  $P = 0.02$ ). No significant differences were found between the two reconstructive methods in terms of intraoperative blood loss (WMD 26.90, 95%CI: -46.54, 100.34;  $P = 0.47$ ), hospital stay (WMD 1.40, 95%CI: -0.17, 2.97;  $P = 0.08$ ), anastomotic leakage (OR 1.26, 95%CI: 0.40, 3.97;  $P = 0.70$ ), stricture (OR 0.94, 95%CI: 0.31, 2.89;  $P = 0.91$ ), dumping symptoms (OR 0.59, 95%CI: 0.32, 1.12;  $P = 0.11$ ) and delayed gastric emptying (OR 0.95, 95%CI: 0.24, 3.74;  $P = 0.94$ ). Results also suggest that B- I reconstruction require shorter operation time (WMD 31.30, 95%CI: 12.99, 49.60;  $P = 0.0008$ ) as compared to R-Y procedure.

### Publication bias

Funnel plot analysis of the studies in the meta-analysis reporting was performed on operation time after DG in RCTs and remnant gastritis in OCS respectively. None of the studies lay outside the limits of the 95%CI, and there was no evidence of publication bias among the studies (Figure 4).

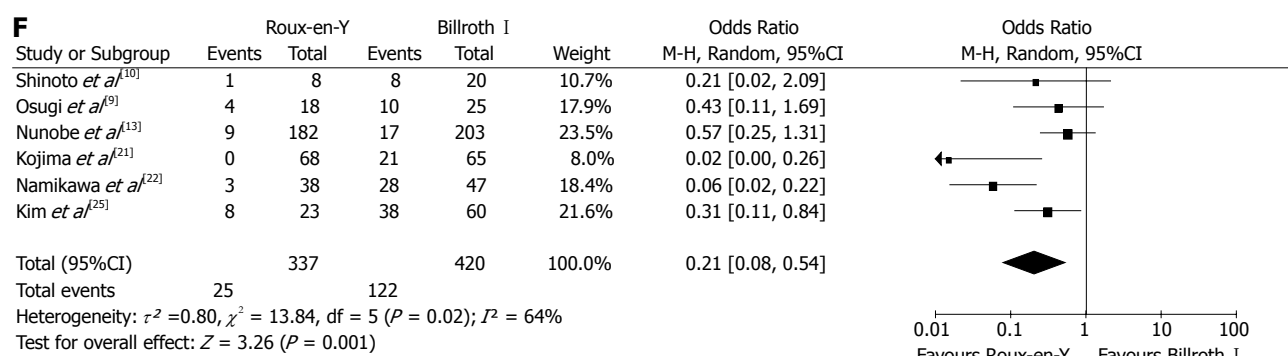
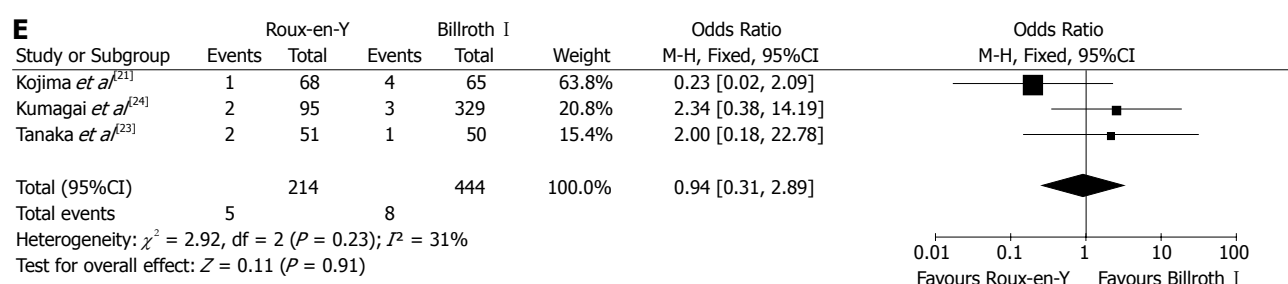
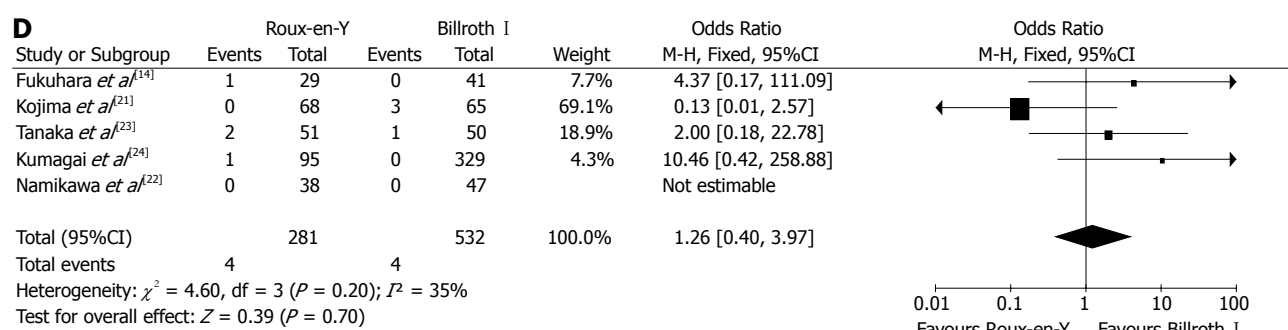
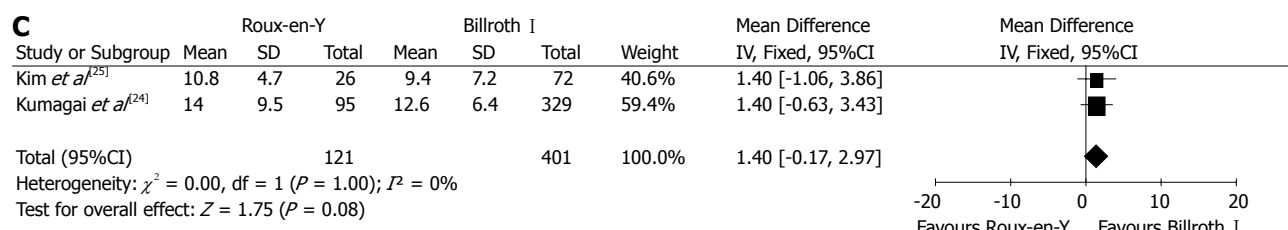
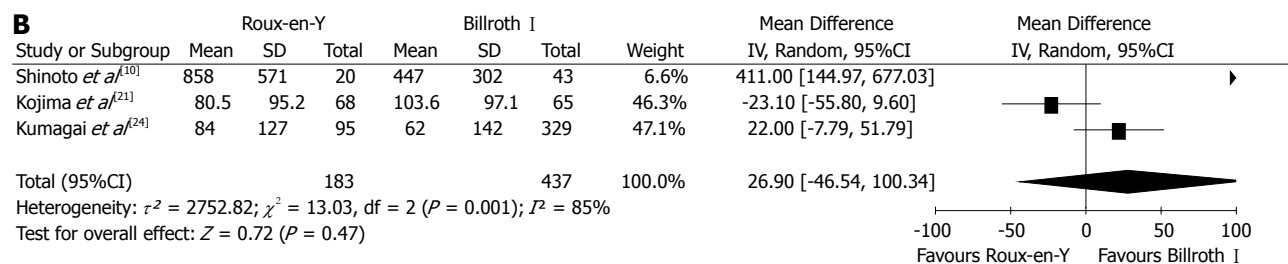
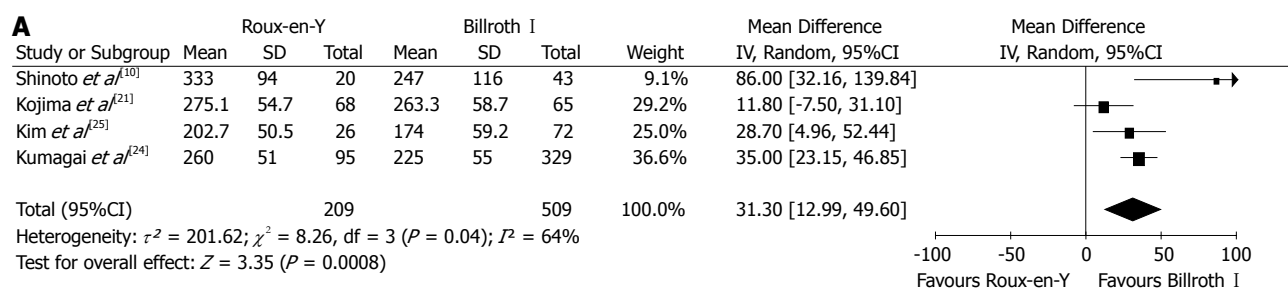
## DISCUSSION

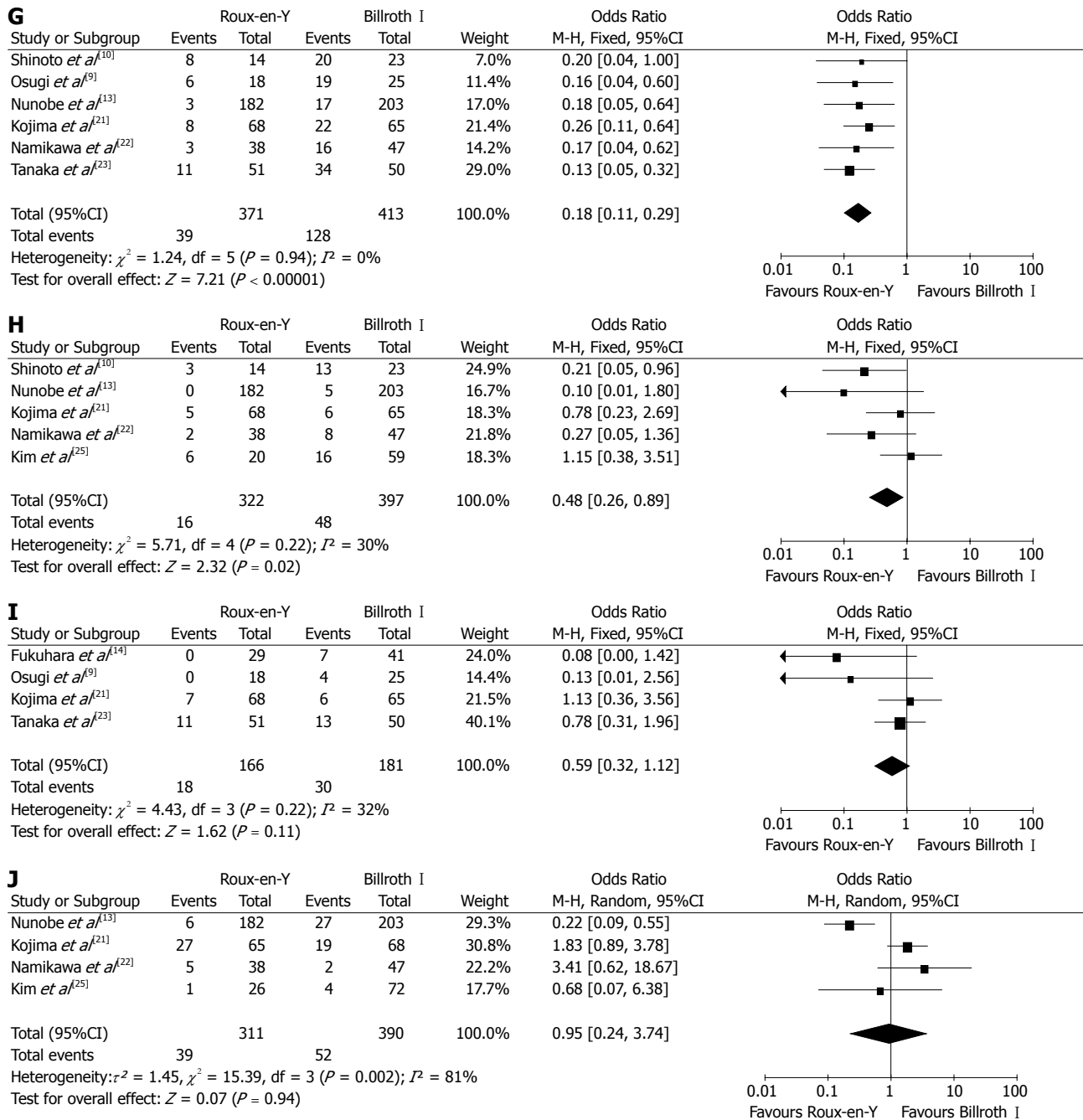
Surgical intervention plays a vital role in the survival of patients with resectable gastric cancer. However, there seems to be lack of consensus within surgeons with regards to the choice of reconstructive procedure after DG. The ideal gastrointestinal reconstruction procedure should minimize postoperative morbidity and improve quality of life<sup>[32]</sup>. In current surgical practice, B- I and R-Y procedures are the commonly used reconstruction techniques following resection of distal stomach. To the best of our knowledge, B- I reconstruction has commonly been employed after DG for gastric cancer due to its simplicity, physiological advantage of allowing food to pass through the duodenum and ease of postoperative endoscopy allowing access to the papilla of Vater<sup>[33,34]</sup>. However, two most common drawbacks of the B- I anastomosis, remnant gastritis and reflux esophagitis, as a consequence of the absence of the pyloric sphincter which allows reflux of duodenal contents into the remnant stomach and esophagus have been well reported<sup>[35]</sup>. Furthermore, unregulated release of chyme into the duodenum results in rapid gastric emptying which manifests as dumping syndrome<sup>[36]</sup>. It is important to note that reflux of duodenal contents into the esophagus is strongly associated with Barrett's esophagus or esophageal cancer and remnant stomach cancer after gastrectomy<sup>[37-39]</sup>.

Traditionally R-Y reconstruction has been the reconstruction method of choice in total gastrectomy<sup>[34]</sup> and is being increasingly used to prevent duodenogastric and gastroesophageal reflux in DG<sup>[10,14,21]</sup>. The potential advantages of improved postoperative quality of life take precedence over the possible increased risk of postoperative complications due to two gastrointestinal anastomoses and increased operating time, when considering R-Y reconstruction.

Based on our analysis, which only includes high qual-





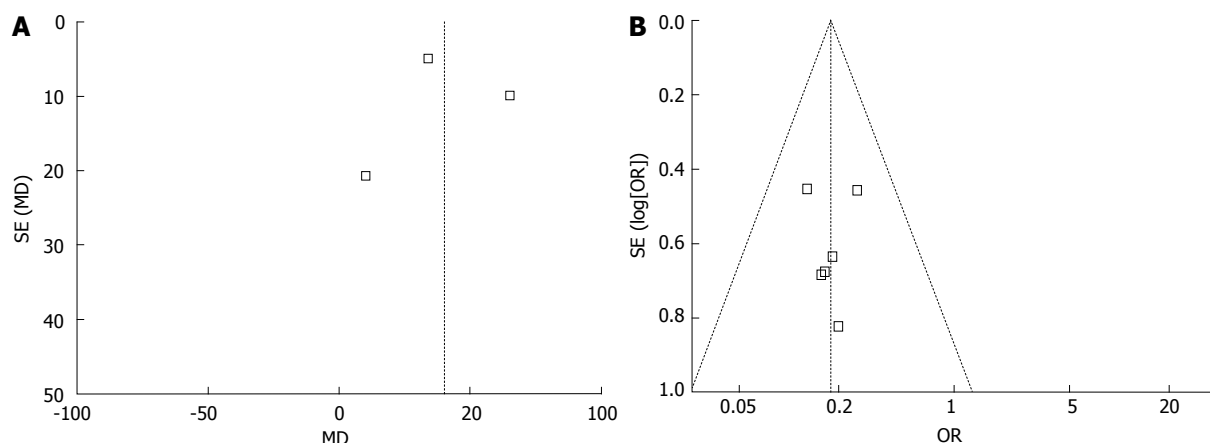


**Figure 3 Roux-en-Y versus Billroth I-observational non-randomized clinical studie comparison.** A: Operation time; B: Intraoperative blood loss; C: Hospital stay; D: Anastomotic leakage; E: Anastomotic stricture; F: Bile reflux; G: Remnant gastritis; H: Reflux esophagitis; I: Dumping symptoms; J: Delayed gastric emptying. Pooled weighted mean difference (WMD) or odds ratio (OR) with 95%CI was calculated using the fixed-or random effects model.

ity RCTs and OCS, we have addressed this issue, to the best of our effort, of the most appropriate gastrointestinal reconstruction following DG. It is important to note that the results come from a small number of RCTs and therefore, have to be interpreted with caution. Overall analyses for RCTs comparing R-Y with B- I reconstruction favor R-Y method in terms of preventing postoperative bile reflux and remnant gastritis, although we found no significant difference in reflux esophagitis between the two groups. The angle of His is significantly larger in patients who undergo B- I reconstruction compared to those with R-Y and this may be a factor contributing

to reduced incidence of reflux symptoms in the latter group<sup>[22,28]</sup>. We may not have detected an existing difference due to the smaller sample size of the included studies. As only one RCT<sup>[20]</sup> evaluated incidences of dumping syndrome, descriptive analysis was used, and no difference was found between the two groups with regards to dumping syndrome.

The operating time was significantly shorter in B- I group compared to R-Y group, which can be explained by the additional anastomosis in R-Y reconstruction. Although it has been previously reported that anastomotic leak is higher in B- I reconstruction, possibly due to ex-



**Figure 4** Funnel plot. A: Operation time-randomized controlled trial; B: Remnant gastritis-observational clinical studies. None of the studies lay outside the limits of the 95% CIs, and there was no evidence of publication bias.

cessive devascularization of duodenal stump and tension on the anastomosis<sup>[20,21,27]</sup>, we found no difference in the rate of anastomotic leak within the two groups. It may be largely due to the use of gastrointestinal stapling devices and the refinement of technique.

Similarly, analysis of pooled data from the OCS, revealed a reduced incidence of bile reflux, remnant gastritis, reflux esophagitis and prolonged operative time in the R-Y reconstruction group. Consequently, based on the above findings, we can conclude that R-Y reconstruction following DG is likely to be superior to B-I reconstruction not only in preventing bile reflux and remnant gastritis, but also reflux esophagitis (OCS analysis only), as it reduces duodenogastric and gastroesophageal reflux<sup>[13,28]</sup>.

We also analyzed data regarding intraoperative bleeding and duration of hospitalization and found no significant difference in either of these parameters within the two groups, although one would expect an earlier recovery of gastrointestinal function in R-Y reconstruction group.

This review does have some limitations and hence the results should be interpreted with a degree of caution. Firstly, most data are extracted from OCS, with fewer RCTs making it difficult to make firm conclusions. In addition, several important outcomes including remnant gastritis, dumping symptoms and delayed gastric emptying have not been reported adequately in the RCTs. It is important to mention that we were unable to analyze important outcomes including quality of life and incidence of gastric carcinoma in the gastric remnant due to lack of available data. We would therefore propose well-designed RCTs with adequate follow-up and emphasis on assessing important outcomes to clarify ambiguities surrounding the use of these reconstruction methods.

In summary, our systematic review demonstrates that RY reconstruction is likely to be more effective in preventing gastroesophageal reflux or duodenogastric reflux as compared to B-I. Furthermore, we have shown that based on results from RCTs and OCS, RY reconstruction may be used safely without increasing anastomotic leak-

age, anastomotic stricture and intraoperative bleeding.

In conclusion, this systematic review points towards some clinical advantages that are rendered by R-Y compared to B-I reconstruction post DG. However there is a need for further adequately powered, well-designed RCTs comparing the same.

## COMMENTS

### Background

Currently, Billroth I (B-I) and Roux-en-Y (R-Y) reconstructions are commonly performed after distal gastrectomy for gastric cancer. However, deciding which of these reconstruction procedures is superior, remains controversial. Therefore, in order to help arrive at a possible consensus, the authors conducted a systematic review and meta-analysis to compare the clinical efficacy and safety of B-I versus R-Y reconstruction following distal gastrectomy (DG) for gastric cancer.

### Research frontiers

In order to compare the safety and effectiveness of the B-I and R-Y reconstructions, operative outcomes including operation time, intraoperative blood loss and postoperative outcomes such as anastomotic leakage and stricture, bile reflux, remnant gastritis, reflux esophagitis, dumping symptoms, delayed gastric emptying and hospital stay were included in this study.

### Innovations and breakthroughs

Although existing randomized controlled trials (RCTs) and retrospective comparative studies have concluded that R-Y reconstruction could prevent gastroesophageal and duodenogastric reflux following DG, there was a need to further assess the clinical advantages of R-Y and B-I reconstruction based on high-level evidence. This meta-analysis reports that the R-Y reconstruction has some clinical advantages in reducing the incidence of bile reflux and remnant gastritis compared to the B-I technique. Also, R-Y reconstruction does not significantly increase postoperative complications.

### Applications

This study shows that R-Y reconstruction following DG for gastric cancer has some clinical advantages compared with B-I reconstruction. However, taking into account the limited number of studies, further adequately powered and well-designed RCTs should be undertaken to investigate the same.

### Terminology

Following distal gastrectomy, three methods, namely, (1) gastroduodenal anastomosis or B-I; (2) gastrojejunal anastomosis or Billroth-II; and (3) R-Y gastrojejunostomy are mainly used for gastrointestinal tract reconstruction.

### Peer review

This is an interesting study, which can point out to some extent, the direction in gastrointestinal tract reconstruction for the future gastrointestinal surgeon.

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## Gastrointestinal sarcoidosis associated with pneumatosis cystoides intestinalis

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### Abstract

A 39-year-old male reported fevers, weight loss, watery loose stools, and decreased visual acuity in his right eye over the prior five years. He was pancytopenic, had an elevated American council on exercise level, total bilirubin, and alkaline phosphatase. Computed tomography revealed massive hepatosplenomegaly and emphysematous lung changes. Liver biopsy showed non caseating granulomas. The patient was diagnosed with extrapulmonary sarcoidosis and was treated with prednisone. The patient symptomatically improved but 5 mo later presented with abdominal pain caused by perforation of the cecum. He underwent a cecectomy and pathology revealed pneumatosis cystoides intestinalis. This represents the first reported association between pneumatosis cystoides intestinalis and sarcoidosis. The etiology of pneumatosis cystoides intestinalis in this case was likely multifactorial and involved both effects of the corticosteroids as well as the advanced

nature of the gastrointestinal sarcoidosis. Furthermore this case has the unique features of emphysematous lung changes and pancytopenia which are uncommon with sarcoidosis.

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**Key words:** Sarcoidosis; Pneumatosis cystoides intestinalis; Pancytopenia; Emphysema; Corticosteroids

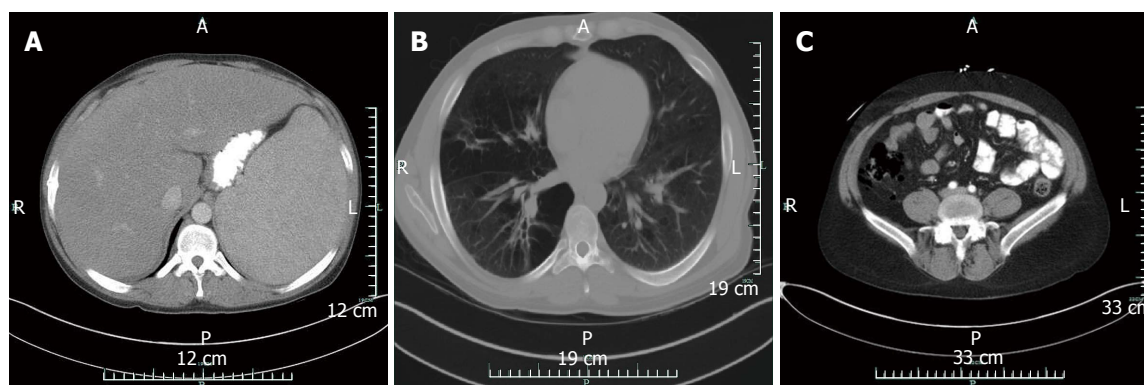
Rahim H, Khan M, Hudgins J, Lee K, Du L, Amorosa L. Gastrointestinal sarcoidosis associated with pneumatosis cystoides intestinalis. *World J Gastroenterol* 2013; 19(7): 1135-1139 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i7/1135.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i7.1135>

### INTRODUCTION

Sarcoidosis is an idiopathic multisystem non-caseating granulomatous disease that has been reported to impact almost any organ. It is more common in African Americans and typically patients present with pulmonary manifestations associated with bilateral hilar adenopathy on chest X-ray. Very rarely does it present with predominantly gastrointestinal (GI) symptoms<sup>[1]</sup>. Pneumatosis intestinalis cystoides is a rare disorder defined as gaseous cysts in the bowel wall. While the etiology remains unclear, it has been associated with multiple conditions including, connective tissue disease, various drugs, colonoscopies, ileal surgeries, and chronic pulmonary disease<sup>[2,3]</sup>. Here we present a case of an individual with GI sarcoidosis who 5 mo after initial presentation was diagnosed with pneumatosis cystoides intestinalis.

### CASE REPORT

A 39-year-old male presented with right lower quadrant abdominal pain that ranged between 5-9/10 severity and was diffusely located in both the right upper and lower



**Figure 1 Computed tomography.** A: Abdomen and pelvis showing massive hepatosplenomegaly at initial presentation; B: Chest at initial presentation demonstrating extensive bilateral emphysematous changes; C: Abdomen revealing pockets of free air consistent with perforation. No evidence of pneumatosis is seen on computed tomography scan. A: Anterior; P: Posterior; R: Right; L: Left.

quadrants of the abdomen. The pain began approximately five years ago and has waxed and waned since. The week prior to admission the pain was significantly worsening. It was particularly aggravated by movement and coughing. Since the onset of symptoms five years ago, he reported watery loose bowel movements after meals, fevers up to 102F, night sweats, a 50lb unintentional weight loss, decreased visual acuity in his right eye and associated photophobia. He also described a chronic cough with occasional dark brown phlegm. He reported waking in the middle of the night coughing. He was initially evaluated multiple times at an outside hospital, but could not remember his specific diagnosis. He has a 15 pack year smoking history and denies any illicit drug use. His prior occupation involved working with copper products. His physical exam revealed severe tenderness over the right side of the abdomen. Liverspan was approximately 25 cm and spleen was easily palpable. The right eye showed an irregular opacity and photophobia.

On presentation he was pancytopenic, with an elevated alkaline phosphatase, gamma glutamyl transpeptidase, total bilirubin and angiotensin converting enzyme (ACE) level. Hemoglobin (Hgb) 13.1 g/dL [normal range (NR) 14.1-17.7 g/dL], platelet count 75 thousand/ $\mu$ L (NR 140-440 thousand/ $\mu$ L), white blood cell (WBC) 2.1 thousand/ $\mu$ L (NR 4.0-10 thousand/ $\mu$ L), alkaline phosphatase 383 IU/ $\mu$ L (NR 45-115 IU/ $\mu$ L), total bilirubin 1.7 mg/dL (NR 0.1-1.3 mg/dL), ACE was 97 U/L (NR 9-67 U/L). Anti-mitochondrial antibody, hepatitis panel, anti-nuclear antibody, human immunodeficiency virus, Epstein-Barr, and rapid plasma reagin serologies were negative. Tuberculin purified protein derivative and sputum acid fast smear were negative.

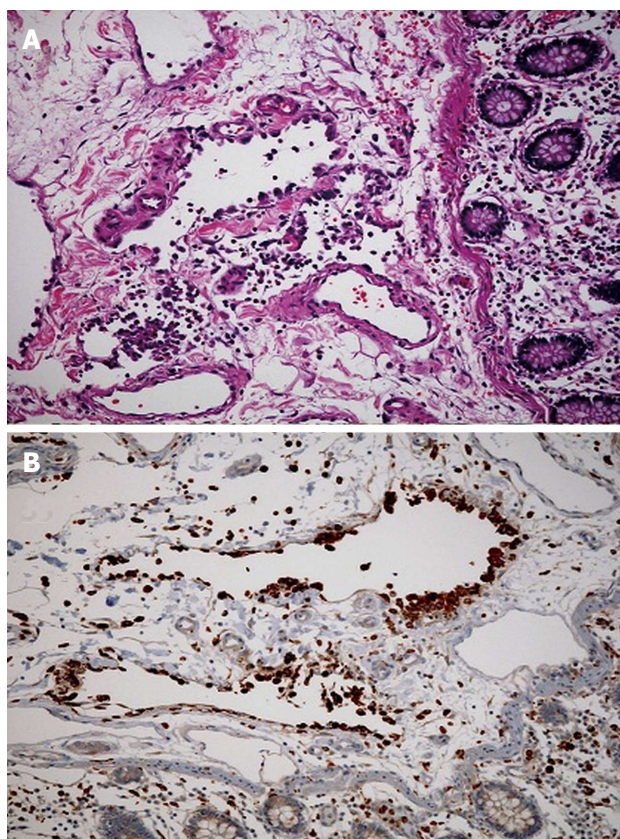
Abdominal computed tomography (CT) revealed a markedly enlarged liver and spleen each measuring 24 cm with distended portal and splenic veins (Figure 1A). Shotty retroperitoneal, upper abdominal and mesenteric lymphadenopathy was noted. CT chest revealed a sub-centimeter enlargement of mediastinal lymph nodes that were pathological in number. Extensive bilateral centrilobular emphysematous changes were noted (Figure 1B). Bone marrow biopsy was normocellular. Immunophenotyping

did not show diagnostic abnormalities of the B- or T-cells. Co-expression of abnormal antigens, antigen deletion, or light-chain clonality was not detected. Outside hospital records from 5 years prior to current admission were obtained. Full body positron emission tomography (PET) scan revealed no foci of abnormal uptake. Hgb was 13.9 g/dL, platelet count 220 thousand/ $\mu$ L, and WBC 4.5 thousand/ $\mu$ L. Alkaline phosphatase 388 IU/ $\mu$ L. Total bilirubin 1.2 mg/dL. Bone marrow biopsy showed hypocellular marrow with mild megaloblastic changes but no evidence of infiltrative changes.

The above findings were most consistent with a chronic inflammatory condition. Chronic inflammatory conditions that can cause hepatic noncaseating granulomas include sarcoidosis, Crohn's disease, primary biliary cirrhosis, Whipple's disease, copper poisoning, lymphomas, secondary syphilis. Given the negative full body PET scan, negative bone marrow and retroperitoneal lymph node biopsy, negative flow cytometry results, and long indolent course lymphoma was highly unlikely. While his previous occupation dealt with copper, symptoms started well before employment there and he did not demonstrate any other signs of copper poisoning. Whipple's disease is more common in European Caucasians and usually does not include ocular manifestations. However, it cannot be definitively excluded without a small bowel biopsy. Primary biliary cirrhosis and syphilis were ruled out through serologies. Crohn's disease is possible but is unlikely to cause such marked hepatosplenomegaly or pancytopenia. An extrapulmonary manifestation of sarcoidosis is the most likely explanation.

The patient was started on a 40 mg prednisone and instructed to follow up in the clinic within a mo. At subsequent follow-ups at outpatient clinic 2 wk, 2 mo, and 3 mo after presentation he was having difficult to control steroid induced diabetes requiring increasing doses of metformin and glipizide. He reported home blood sugar measurements ranged from 100-250 mg/dL. He had two separate hospitalizations for hyperglycemia 1 mo and 4 mo after presentation. At each visit his prednisone dose was being tapered. At 4 mo follow up at outpatient





**Figure 2** Pathology of the resected tissue revealed portions of large bowel with submucosal and mucosal hemorrhage and submucosa with irregular spaces lined with histiocytes (CD68 positive and CD31 negative) consistent with pneumatosis cystoides intestinalis. A: Cecum specimen stained with hematoxylin and eosin demonstrates partially endothelialized cystic spaces in the submucosa lined by eosinophilic cells characteristic of pneumatosis intestinalis; B: Immunohistochemical staining for CD 68 which is found in cytoplasmic granules of cells within the monocyte/macrophage lineage confirms presents of histiocytes lining cystic spaces.

clinic the dose was 25 mg. The second hospitalization occurred at an outside hospital 4.5 mo after initial presentation. On discharge the patient reported he was told to increase the dose of glipizide as well as to increase his prednisone dose from 25 mg to 40 mg daily. Overall he was reporting decreased distension and abdominal discomfort. ACE level measured at outside hospital after 4 mo of steroid therapy was down to 29 U/L (97 U/L at initial presentation).

Five mo after initial presentation he came to our institution with headache, dizziness, abdominal pain, and nonbloody diarrhea 1 d. He reported the abdominal pain and discomfort were approximately baseline for him. He was afebrile and normotensive. His abdomen was tender to palpation right greater than left with guarding and rebound. Fecal occult blood was negative. He was found to have blood glucose of 623 mg/dL. Admission labs demonstrated improved pancytopenia, decreased alkaline phosphatase, but an elevated lactate [lactate 5.6 mmol/L (normal 0.5-2.2 mmol/L), Hgb (17.6 g/dL), platelets (114 thousand/ $\mu$ L), WBC (6.2 thousand/ $\mu$ L), alkaline phosphatase (223 IU/L)]. CT of the abdomen revealed pockets of free air distributed circumferentially along

the cecum consistent with perforation (Figure 1C). He was taken to the OR for exploratory laparotomy requiring cecectomy and creation of ileostomy and mucous fistula. In the operating room there was no identified perforation externally, but a 2 cm  $\times$  2 cm defect within the cecum that did not penetrate the serosal surface of the bowel. The mucosa was hemorrhagic and flattened. Pathology of the resected tissue revealed portions of large bowel with submucosal and mucosal hemorrhage and submucosa with irregular spaces lined with histiocytes (CD68 positive and CD31 negative) consistent with pneumatosis cystoides intestinalis (Figure 2).

## DISCUSSION

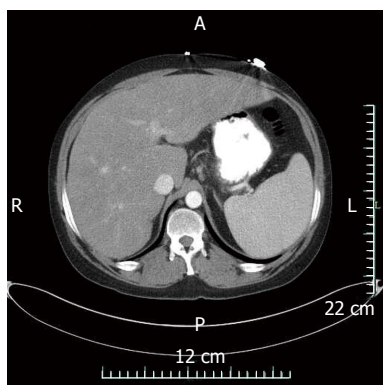
Sarcoidosis is an idiopathic multisystem non-caseating granulomatous disease that has been reported to impact almost any organ. It is more common in African Americans and typically patients present with pulmonary manifestations associated with bilateral hilar adenopathy on chest X-ray<sup>[1]</sup>. Other organs frequently involved include skin, lymph nodes, and ocular manifestations. The diagnosis is established by exclusion of other possibilities and can be supported by characteristic physical findings and biopsy showing noncaseating granulomas. ACE levels are elevated in 10% of chronic sarcoidosis and 60% of acute sarcoidosis<sup>[1]</sup>.

The patient's chief complaint related to the abdominal distension and pain secondary to hepatosplenomegaly. Abdominal pain is an infrequent chief complaint in sarcoidosis however, both the liver and spleen have been reported to be involved<sup>[4]</sup>. Hepatomegaly has been reported to be as high as 21% of patients clinically and as many as 13% of patients with liver involvement had involvement independent of any typical pulmonary manifestations<sup>[4]</sup>. Splenomegaly has been reported by physical exam in 5%-14% with hypersplenism seen in 15% of those with splenic involvement<sup>[4]</sup>. While biopsy specimens from the patient presented are not available given the degree of splenomegaly and pancytopenia one could hypothesize involvement.

The patient described presented with pancytopenia likely secondary to hypersplenism. He was severely thrombocytopenic and leukopenic and mildly anemic. Cytopenias have been frequently described in sarcoidosis. However, to our knowledge significantly fewer reports describing pancytopenia. One report describes thrombocytopenia and leukopenia secondary to hypersplenism a second report notes pancytopenia with splenomegaly both were resolved with splenectomy<sup>[5,6]</sup>. This indicates a potential therapeutic role for the patient described here, but repeat CT after 5 mo on steroids revealed dramatic improvement in spleen size (Figure 3).

Pneumatosis cystoides intestinalis is a rare disorder defined as gas within the bowel wall. The presentation can range from being asymptomatic to acute abdominal pain. The mechanism of pneumatosis cystoides intestinalis is multifactorial. Current theories propose luminal gas which could be pulmonary or bacterial in origin dis-





**Figure 3** Computed tomography abdomen with contrast demonstrating marked reduction in spleen size and moderate reduction in hepatomegaly after 5 mo of steroid treatment. A: Anterior; P: Posterior; R: Right; L: Left.

sect through mucosa which has been compromised. Mucosa may be compromised by a number of mechanisms including immunologically or physically<sup>[7]</sup>.

While typically diagnosed *via* CT scan<sup>[2]</sup> certain cases where the pneumatosis is very subtle the finding may be missed and air contrast enema may be useful<sup>[8,9]</sup>. Furthermore, when diagnosed *via* CT, the majority of patients can be managed nonoperatively as exploratory laparotomy is likely to be of benefit to only a subset of patients. Those patients include those with abdominal distension, peritonitis, and lactic acidemia<sup>[10]</sup>. The patient described here represents one of these subtle cases as he was only found to have pneumatosis on pathology. He clearly was in need of operative management given the pneumoperitoneum, abdominal distension, and elevated lactic acid.

Pneumatosis cystoides intestinalis has been linked to a number of conditions in the past. One of which is chronic obstructive lung disease<sup>[2,3]</sup>. Pulmonary function tests obtained 4 mo after presentation at an outside institution revealed moderate obstructive lung disease with abnormal diffusion capacity [forced expiratory volume in 1 second (FEV1)/forced vital capacity = 70% predicted, FEV1 = 69% predicted, single breath diffusion Dsb 38% predicted]. Typically sarcoidosis most commonly affects the lungs, but the typical radiographic presentation was absent. On chest X-ray bilateral and mediastinal lymphadenopathy (75%) and pulmonary infiltrates (50%) are the most common presentations<sup>[1]</sup>. This usually occurs in an upper lobe and nodular predominance. On CT scan typical features include bilateral perihilar opacities, bilateral symmetric hilar or mediastinal lymphadenopathy, fibrotic changes such as reticular opacities, architectural distortion, or bronchiectasis<sup>[1]</sup>. The patient presented had severe emphysematous changes which are rarely associated with sarcoidosis. Prior case reports have documented emphysema in patients with sarcoidosis though the mechanism is unclear<sup>[11,12]</sup>. While he does have a 15 pack year hx it would be unlikely all lung abnormalities are due to past tobacco at only the age of 39, though this does confound the finding.

Pneumatosis cystoides intestinalis has been very frequently linked to medications. Corticosteroids have been

reported most often. Many of the other conditions, pneumatosis cystoides intestinalis has classically been linked to are treated with steroids as well, including connective tissue disease and some autoimmune conditions<sup>[2,3,13,14]</sup>. The hypothesized mechanism suggests the immunosuppressive effects of steroids results in depletion of the lymphoid tissue within the Peyer's patches, this leads to mucosal disruption allowing intraluminal gas diffusion<sup>[15,16]</sup>. One review indicates that 33% of patients with pneumatosis intestinalis had received prior steroid treatment. Another 32% had received either chemotherapy or methotrexate<sup>[15,17]</sup>. This hypothesis is supported by the fact that other immunosuppressive or cytotoxic drugs have also been associated with pneumatosis cystoides intestinalis. This includes but is not limited to sunitinib, cisplatin, irinotecan, and docetaxel, methotrexate<sup>[17-20]</sup>.

Alternative mechanisms have been described for other drugs that have been implicated in pneumatosis cystoides intestinalis. The chemotherapeutic agents described above have not only been suggested to contribute to the development of pneumatosis cystoides intestinalis *via* their immunosuppressive effects but also through their apoptotic effects on rapidly dividing cells resulting in compromise of mucosal integrity. Vascular endothelial growth factor and epidermal growth factor inhibitors are hypothesized to damage the microvasculature of the intestinal wall resulting in compromise of mucosal integrity<sup>[19,21]</sup>. Alpha glucosidase inhibitors, miglitol and acarbose, inhibit the absorption of carbohydrates. It has been suggested that digestion of these carbohydrates by intestinal flora result in gas production. The increased intraluminal pressure could potentially lead to dissection through the mucosa<sup>[15,22,23]</sup>.

The patient was on a steroid taper, but was increased from 25 mg to 40 mg by an outside hospital for reasons that are unclear 1 mo prior to perforation. The 5 mo course of steroids coupled with the recent increased dosage thus could have played a significant role in the pathogenesis through immunosuppression as detailed above. However, given the patients improvement in symptoms and ACE level it seems the initial course of tapering the dosage as symptoms improved was the best course of action.

The patient described here likely had a number of factors that could have additively contributed to the development of pneumatosis cystoides intestinalis. The steroid treatment coupled with his baseline pancytopenia clearly placed him in an immunosuppressed state. The massive hepatomegaly and respiratory disease may have lead to increased intraabdominal pressure producing strain on the mucosal walls. We may also speculate that the 5 year history of untreated GI sarcoidosis may have further weakened the intestinal walls through granulomatous inflammation and disruption of normal architecture. Cumulatively, this created an environment where pneumatosis intestinalis could readily develop.

The described case represents a unique presentation of the difficult to recognize and diagnose GI sarcoidosis. The initial findings of pancytopenia and emphysematous

changes in the lungs are typically not found in sarcoidosis. Furthermore this is the first reported association between pneumatosis intestinalis and sarcoidosis though the exact mechanism of pneumatosis cystoides intestinalis is unclear and likely multifactorial as in many such cases.

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## Transjugular intrahepatic portosystemic shunt in refractory chylothorax due to liver cirrhosis

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### INTRODUCTION

Chylothorax is defined as a pleural effusion containing chylomicrons. Triglyceride levels of at least 110 mg/dL have been found to reliably indicate chylothorax<sup>[1]</sup>, originating from a lesion in the lymphatic vessels, mostly the thoracic duct. In about 40% of patients with chylothorax, these lesions are caused by trauma or surgery, whereas in about 30%, a malignant tumor is found. The remaining cases are attributed to a variety of conditions, including congenital, infectious and cardiac diseases, or are considered to be idiopathic<sup>[2]</sup>. Chylothorax rarely occurs in patients with liver cirrhosis. In such cases, concomitant ascites is usually present<sup>[3]</sup>. We report on the effective treatment of massive chylothorax in a female patient with liver cirrhosis but no ascites by placement of a transjugular intrahepatic portosystemic shunt (TIPS).

### CASE REPORT

The 59-year-old woman was referred to our university hospital due to chylothorax which could not be sufficiently managed in a peripheral hospital. Since 2008, a right sided pleural effusion had to be drained occasionally despite diuretic therapy. In recent weeks, however, the pleural effusion had turned milky and had to be drained more frequently. Three weeks prior to admission, nearly 1.5 L/d had to be removed (Figure 1). The patient presented no further symptoms apart from dyspnea. Her past medical history included diagnosis of alcoholic liver cirrhosis (Child B), diabetes mellitus type II and resection of a local ovarian carcinoma 15 years ago. The exact tumor staging was unknown. The patient had received adjuvant chemotherapy and the carcinoma has been in remission since then. One year ago, she was treated for bleeding gastric ulcer related to infection with *Helicobacter pylori*. There was no history of trauma.

### Abstract

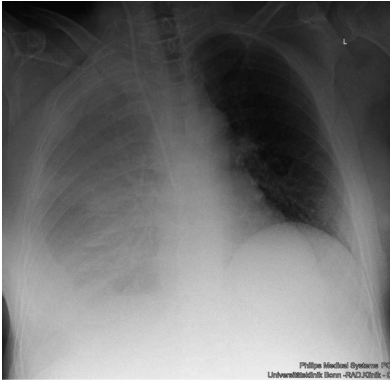
A pleural effusion containing chylomicrons is termed chylothorax and results from leakage of lymph fluid into the pleural cavity. We report on the case of a 59-year-old woman with severe dyspnea due to a large chylothorax. She was known to have liver cirrhosis but no ascites. There was no history of trauma, cardiac function was normal and thorough diagnostic work-up did not reveal any signs of malignancy. In summary, no other etiology of the chylothorax than portal hypertension could be found. Therapy with diuretics as well as parenteral feeding failed to relieve symptoms. After a transjugular intrahepatic portosystemic shunt (TIPS) had successfully been placed, pleural effusion decreased considerably. Eight months later, TIPS revision had to be performed because of stenosis, resulting in remission from chylothorax. This case shows that even in the absence of ascites, chylothorax might be caused by portal hypertension and that TIPS can be an effective treatment option.

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**Key words:** Chylothorax; Cirrhosis; Liver; Portal hypertension; Transjugular intrahepatic portosystemic shunt

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**Figure 1** Chest X-ray before transjugular intrahepatic portosystemic shunt placement, showing a large right-sided pleural effusion.



**Figure 2** Angiogram after transjugular intrahepatic portosystemic shunt placement, the catheter tip is in the portal vein.

Apart from the signs of right-sided pleural effusion, some spider naevi and a small umbilical hernia, physical examination was unremarkable. Blood analysis showed slightly elevated bilirubin (1.2 mg/dL, reference range up to 1 mg/dL), increased gamma-glutamyl transferase (112 U/L, reference range up to 38 U/L), international normalized ratio (of prothrombin time) above normal (1.3) and decreased serum albumin (31 g/L, reference range 35-52 g/L). Model for End-stage Liver Disease score was 10. Mild hyperglycemia was due to diabetes mellitus. Urine analysis was unremarkable. Analysis of the pleural fluid showed 95 polymorphonuclear cells/ $\mu$ L, 294 mononuclear cells/ $\mu$ L, low albumin of 4.2 g/L, no cholesterol, but markedly increased triglycerides (386 mg/dL). Bacterial cultures were negative, microscopic examination did not reveal malignant cells. No tumor was detected in a computed tomography (CT) scan carried out at the referring hospital. Cardiac evaluation by echocardiography and transthoracic echocardiography revealed no signs of heart failure or pulmonary hypertension. Abdominal ultrasound showed a liver morphology consistent with cirrhosis and concomitant splenomegaly, but neither portal vein thrombosis nor signs of hepatocellular carcinoma nor ascites were present. To further characterize the degree of portal hypertension, we performed endoscopy of the upper gastrointestinal tract. Esophageal varices grade II were detected.

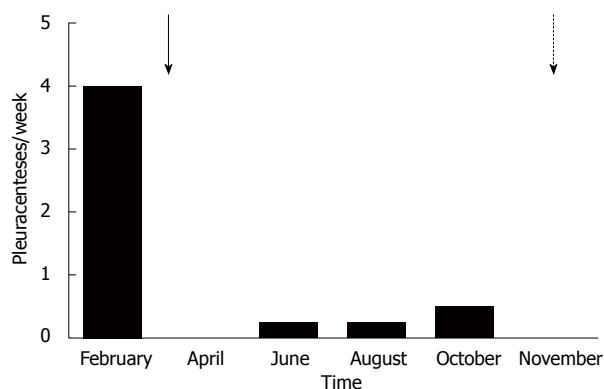
In summary, we confirmed the diagnosis of chylothorax with a daily production of 2-4 liters. However, it was uncertain whether this was caused by liver cirrhosis. As diuretic therapy failed to drain the chylothorax, we put the patient on parenteral feeding for a total of nine days to reduce the flow in the lymphatic ducts and to promote healing of a suspected lymphatic lesion. As expected, the milky aspect of the pleural effusion vanished, but the daily amount of secretion from the pleural drainage increased even further (about 1.5-3 L/d). Since high renin blood levels are often associated with decompensated portal hypertension<sup>[4]</sup>, we determined renin blood levels, revealing a value of 1680  $\mu$ U/mL (reference range up to 46  $\mu$ U/mL) in conjunction with an elevated level of aldosterone of 946 pg/mL (reference range up to 310 pg/mL). Thus, we proposed TIPS as treatment option and this was placed successfully in March 2010 (Figure

2). Fifteen days later, the pleural drain could be removed; another week later, the patient could be dismissed with a small, stable pleural effusion under diuretic medication. During the following months, the pleural effusion had to be drained about once per month. TIPS revision due to stenosis had to be performed by inserting a new stent in November 2010. Since then, no further pleural drainage was needed (Figure 3). After TIPS placement, the patient had several episodes of hepatic encephalopathy grade I - II according to the West Haven criteria, but responded to treatment with lactulose and rifaximin. Levels of bilirubin did not increase significantly after the intervention. To date, more than two years after TIPS placement, the patient is still without recurrence of chylothorax (Figure 4).

## DISCUSSION

Liver cirrhosis, together with portal hypertension is a rare cause of chylothorax<sup>[3]</sup>. Diagnosis was difficult in this patient because she presented with almost normal laboratory values and without ascites. However, tiny amounts of abdominal fluid had been present in the past. Conservative management of chylothorax failed. To the best of our knowledge, TIPS as successful treatment of chylothorax in cirrhotic patients has been described before in only two cases: in one patient with traumatic injury of the thoracic duct<sup>[5]</sup> and in a second patient with concomitant refractory ascites<sup>[6]</sup>. High renin levels in our patient suggested that activation of the renin angiotensin aldosterone system (RAAS), causing sodium and fluid retention, was due to portal hypertension, complicated by the leakage of chyle in the pleural cavity. This activated RAAS argued against idiopathic etiology, which is described in 10% of patients with chylothorax. Indeed, renin values in our patient decreased considerably from 1680 to 317  $\mu$ U/mL after TIPS placement, along with regression of excessive chyle formation. Other etiologies of chylothorax were not very plausible: there was no history of recent trauma or surgery and a malignant process - bearing the history of ovarian cancer in mind - was improbable due to negative CT scan and pleural fluid cytology. Furthermore, the patient developed no malignancy during the follow-up of more than two years. In the end, placement of TIPS improved the quality of life



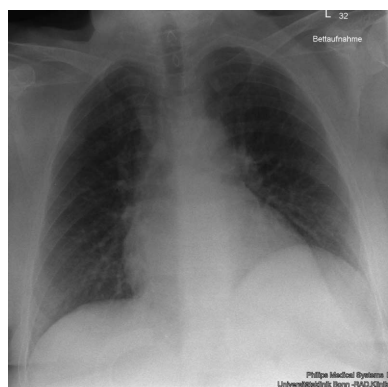


**Figure 3** Need for pleurocentesis over time. There is a clear drop after transjugular intrahepatic portosystemic shunt (TIPS) placement in March (arrow) and no need for further pleurocentesis after TIPS revision in November (dotted arrow).

of this patient enormously. After failure of conservative treatment, the main alternative to TIPS would have been surgical procedures, such as pleurodesis or ligation/embolization of the thoracic duct<sup>[7]</sup>, which, however, do not address portal hypertension as underlying disease. Fasting and parenteral nutrition have been described as effective in chylothorax particularly following surgery or trauma. In most other forms, lymphatic obstruction or increased venous pressure in the superior vena cava cause lymphangiectasia and chylothorax<sup>[7]</sup>. In these cases, parenteral feeding is not effective. Somatostatin has been used in chylothorax of various etiologies with some success<sup>[8]</sup>. Due to its characteristic contents, drainage of a chylothorax can lead to malnutrition and immunosuppression caused by loss of lipids, immunoglobulins and lymphocytes. However, this characteristic content seems to reduce the risk of infection of a pleural drain<sup>[9]</sup>.

The success of the TIPS placement in our patient confirms the hepatic origin of the chylothorax. It has been suggested that in liver cirrhosis, chyle flow increases substantially due to increased formation of hepatic lymph and due to portal hypertension. Since drainage into the venous system is limited by a valve at the junction of the thoracic duct and the subclavian vein, pressure in the lymphatic vessels is increased, leading to an elevated risk of spontaneous rupture<sup>[10]</sup>. Thus, in our patient, such a spontaneous leak might have persisted as long as portal hypertension was high, maintaining the pleural effusion *via* small gaps in the diaphragm as in hepatic hydrothorax, which occurs in about 5%-12% of liver cirrhosis patients<sup>[11]</sup>. A recent review described a clinical response rate of about 70% in 198 patients with hepatic hydrothorax after TIPS placement. However, controlled studies are still missing. No case of chylothorax was described in these patients<sup>[12]</sup>.

In summary, this case shows that massive chylothorax can develop in patients with otherwise well compensated liver cirrhosis and that it can be treated effectively



**Figure 4** Chest X-ray during follow up without pleural effusion.

by TIPS placement.

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## Cytomegalovirus-associated gastric ulcer: A side effect of steroid injections for pyloric stenosis

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### INTRODUCTION

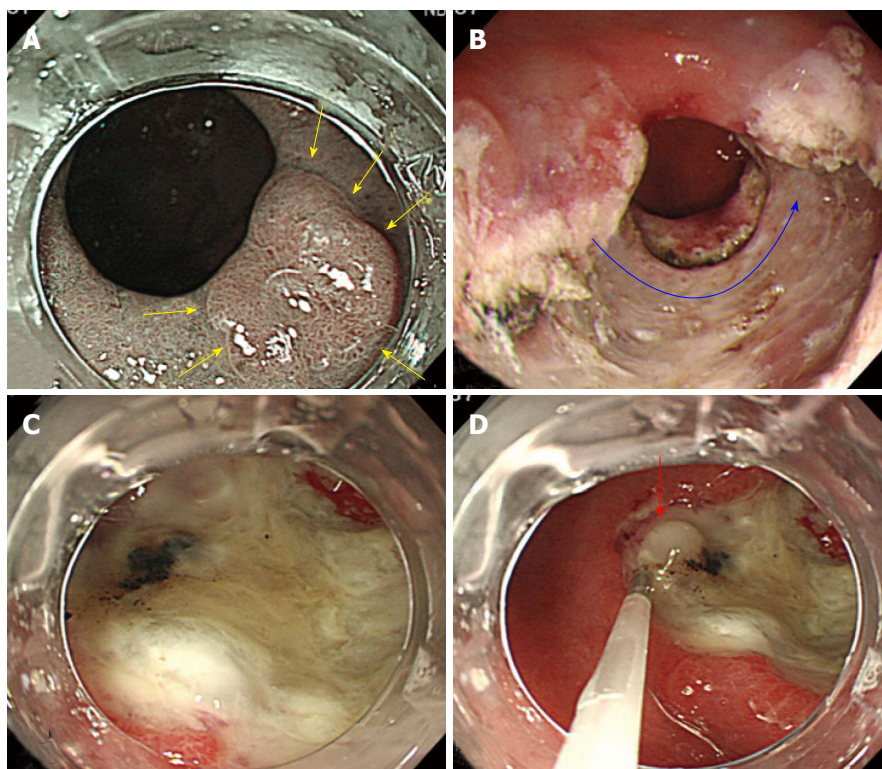
Endoscopic submucosal dissection (ESD) for early gastric cancer was developed to resect larger lesions *en bloc*<sup>[1-4]</sup>. ESD has an advantage over endoscopic mucosal resection in that it enables *en bloc* tumor removal<sup>[5-8]</sup>. However, ESD creates a large artificial ulcer that can lead to gastric stenosis<sup>[9]</sup>. In recent years, the local injection of triamcinolone acetate (TA) has reportedly prevented post-ESD esophageal stricture, pyloric stenosis, and deformity following large ESDs because TA promotes the formation of granulation tissue at an early stage in the healing process, which leads to gastric mucosa regeneration<sup>[10-12]</sup>. However, because of its long-acting nature, TA can induce long-term local immunosuppression and can cause subsequent adverse events. We report a case of cytomegalovirus (CMV) ulcer formation that occurred only at the local TA injection site. This is the first case report of a side effect of local TA injection after treatment of an ESD ulcer floor in a non-compromised host.

### CASE REPORT

A 68-year-old man underwent ESD to treat early-stage gastric cancer that was located over the pylorus (Figure

### Abstract

The local injection of triamcinolone acetate (TA) is effective in preventing pyloric stenosis and deformity following large endoscopic submucosal dissection (ESD). However, because of its long-acting nature, TA can induce long-term local immunosuppression and subsequent adverse events. We report a case of a cytomegalovirus (CMV) ulcer that formed only at the TA local injection site. A 68-year-old man underwent ESD to treat early gastric cancer that formed over the pylorus. The lesion extended to the duodenum, and an artificial ulcer covered more than two-thirds of the circumference of the pylorus. To prevent pyloric stenosis, TA was locally injected into the ulcer floor. On day 12, a deeper ulcer 10 mm in diameter was discovered in the center of the post-ESD ulcer. Biopsies revealed large cells with intranuclear inclusion bodies, which stained positive for the anti-CMV antibody. Local TA injections are useful, however, CMV ulcer might occur as adverse events.



**Figure 1** Endoscopic findings of tumor, post-endoscopic submucosal dissection ulcer and triamcinolone acetate injection. A: An narrow band imaging endoscopic image reveals a flat, early gastric cancer lesion extending over the gastric outlet to the pylorus (yellow arrows); B: A post-endoscopic submucosal dissection artificial ulcer covering two-thirds of the circumference of the pylorus (blue curved arrow); C: The ulcer floor covered by a thick layer of white moss; D: Triamcinolone acetate (2 mL) was injected locally at each site (red arrow).

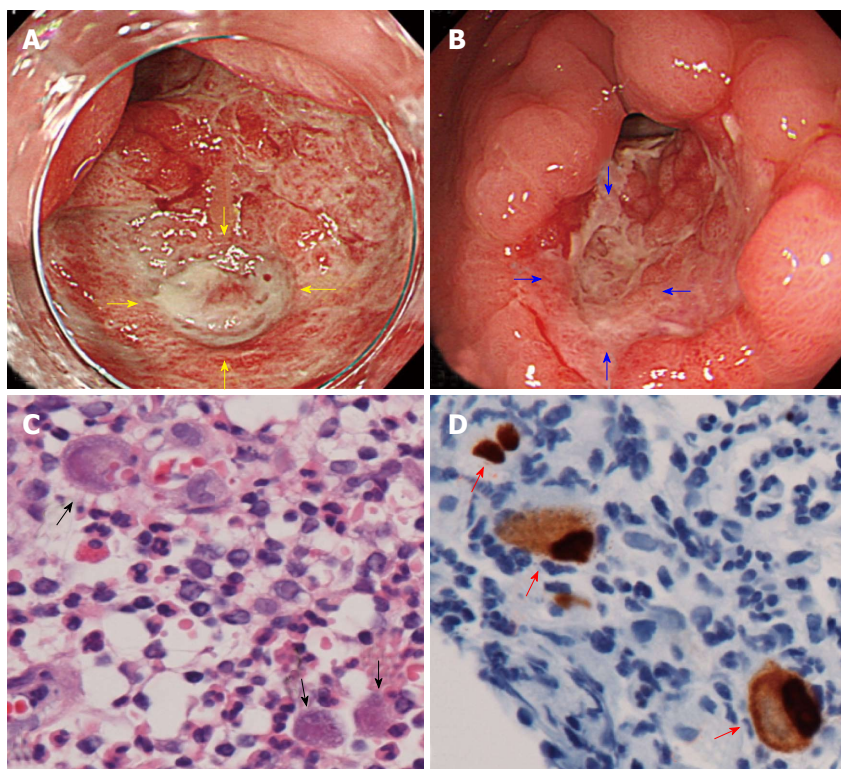
1A). The lesion partially extended to the duodenum, and an artificial ulcer that formed after dissection covered over two-thirds of the pylorus circumference (Figure 1B). As routine pre-ESD examination, we conducted serology test, electrocardiogram, respiratory function test, abdominal ultrasound examination and computed tomography. These results indicated no underlying disease. To prevent pyloric stenosis, TA was locally injected into the ulcer, which was covered with white moss (Figure 1C) in 5-mm intervals [0.2 mL (2 mg)] at each site, on postoperative day 5 (Figure 1D). On day 12, abundant granulation tissue had formed over the ulcer, but another deeper ulcer approximately 10 mm in diameter was discovered centered in the post-ESD ulcer (Figure 2A). Biopsies that were conducted from the margin of the deeper ulcer revealed large cells with intranuclear inclusion bodies (Figure 2C) that stained positive for anti-CMV antibody staining (Figure 2D). As the patient wasn't a compromised host and had no other underlying disease, we thought the CMV activity of the ulcer floor was limited and focal. The CMV ulcer was occurred under focal immunosuppressive condition by TA. We considered after TA effect would subside about for 14 d, the CMV activity would decrease and the ulcer healed. We conducted frequent follow up endoscopy at POD12, 20 and 30, and confirmed negative conversion of CMV by serology test and histopathological examination. The deeper ulcer improved gradually (Figure 2B), and on day 20, the biopsies were negative for anti-CMV antibody

staining. The post-ESD artificial ulcer healed without any pylorus stricture.

## DISCUSSION

Some clinical studies have recommended administration of oral prednisolone<sup>[10]</sup> and local TA injection into post-ESD artificial ulcers<sup>[11]</sup> in order to prevent severe esophageal stenosis. The beneficial effects of this procedure were introduced at the Conference of Japan Gastroenterological Endoscopy in April 2009, and some clinical trials reported on the efficacy of local TA injections and oral prednisolone administration. After these reports, we reported that local TA injection into the floor of a post-ESD artificial gastric ulcer promotes the formation of granulation tissue in an early stage of the healing process, which leads to regenerated gastric mucosa without mucosal convergence or gastric deformity<sup>[12]</sup>. As our previous report, we conducted local steroid injection into post-ESD artificial ulcers to 21 patients and analyzed them according to the protocol, there were no complications. So, this case was the first case of CMV-associated ulcer development related to the side effects of local steroid injection following ESD. Pharmacologically, TA modulates the wound-healing process for post-ESD ulcers by suppressing inflammatory cell infiltration and fibrosis. This wound healing is caused by decreasing the procollagen-proline dioxygenase or prolyl hydroxylase levels. Decreasing the levels of these enzymes reduces the tissue





**Figure 2** Endoscopic findings of cytomegalovirus associated ulcer and microscopic examination. A: Artificial ulcer on postoperative day 12, showing the formation of abundant granulation tissue and a 10-mm-deep ulcer at the center of the granulation tissue (yellow arrows); B: The healing process of the deep ulcer (blue arrows) on postoperative day 15; C: A biopsy from the deeper ulcer margin revealed large cells with intranuclear inclusion bodies (black arrow, HE staining,  $\times 600$ ); D: Large cells with intranuclear inclusion bodies stained positive for anti-cytomegalovirus (CMV) antibodies (red arrows, anti-CMV antibody immunohistochemical staining,  $\times 600$ ).

collagen component<sup>[13]</sup>; in this way, the local TA injection promoted the formation of flat and sufficient granulation tissue without fibrotic contraction. However, no infections or adverse effects were reported following local steroid injection into the post-ESD artificial esophageal or gastric ulcer floor. Subclinical CMV infection is high among Japanese infants (about 90%). Latent infection, which is established in the granulocytes and monocytes, can be reactivated by administering potent immunosuppressants, such as steroids<sup>[14]</sup>. Although the CMV ulcers generally occurred in compromised hosts, in our case, the formation of a CMV ulcer was likely caused by an inflammatory reaction in the post-ESD ulcer and reactivation of the latently infected granulocytes and monocytes, which had migrated to phagocytize the necrotic and granulation tissues on the ulcer floor. Ulcer healing occurred after 14–21 d when the TA effects had subsided. The local injection of TA is effective in preventing post-ESD esophageal stricture or pyloric stenosis; however, after the CMV is reactivated, the long-acting nature of TA may delay the healing process. CMV is recognized an important pathogen of severe infections in immunocompromised hosts, and causes CMV mononucleosis with multi-organ involvements. In general, CMV-related ulcers might make multiple and deeper lesions in digestive tract in upper gastrointestinal endoscopy<sup>[15]</sup>.

In this case, the ulcer located at the center of the post-ESD artificial ulcer floor which was limited and lo-

cal area under focal immunosuppressive condition by TA.

In conclusion, the local steroid injection might be an etiological factor for CMV-associated ulcers. We advise that clinicians observe the ulcer floor following TA injections and quickly treat CMV-associated ulcers with ganciclovir if needed.

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## Isolated fever induced by mesalamine treatment

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### Abstract

Adverse reactions to mesalamine, a treatment used to induce and maintain remission in inflammatory bowel diseases, particularly ulcerative colitis, have been described in the literature as case reports. This case illustrates an unusual adverse reaction. Our patient developed an isolated fever of unexplained etiology, which was found to be related to mesalamine treatment. A 22-year-old patient diagnosed with ulcerative colitis developed a fever with rigors and anorexia 10 d after starting oral mesalamine while his colitis was clinically resolving. Testing revealed no infection. A mesalamine-induced fever was considered, and treatment was stopped, which led to spontaneous resolution of the fever. The diagnosis was confirmed by reintroducing the mesalamine. One year later, this side effect was noticed again in the same patient after he was administered topical mesalamine. This reaction to mesalamine seems to be idiosyncratic, and the mechanism that induces fever remains unclear. Fever encountered in the course of a mesalamine treatment in ulcerative colitis must be considered a mesalamine-induced fever when it cannot

be explained by the disease activity, an associated extraintestinal manifestation, or an infectious etiology.

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**Key words:** Mesalamine; 5-aminosalicylic acid; Side effects; Adverse reactions; Fever

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### INTRODUCTION

Mesalamine is a well-known treatment for inflammatory bowel diseases (IBDs), particularly ulcerative colitis (UC), in which it is used for remission induction and maintenance therapy. Mesalamine is considered a safe drug, but some side effects have been described in the literature. We report a case of mesalamine-induced isolated fever in a patient with UC.

### CASE REPORT

A 22-year-old-man presented with a 1 wk history of apyretic acute bloody diarrhea, which was treated with 500 mg of metronidazole three times a day (TID) without clinical improvement. At this stage, flexible sigmoidoscopy showed diffuse hyperemic mucosa with few erosions. The biopsies indicated infectious colitis. The blood analysis and stool exam were normal. Treatment with 500 mg of ciprofloxacin twice a day and 500 mg of metronidazole TID was prescribed. Ten days later, the patient's clinical condition worsened, with passage of 5 stools per day with mucus and blood. A complete colonoscopy was performed, which demonstrated diffuse pancolitis with superficial ulcerations, loss of vascular

pattern and easy contact bleeding from the mucosa. The terminal ileum was normal. The colonic biopsies were consistent with UC. The blood analysis at this time was also unremarkable except for the C reactive protein (CRP) level, which was 17 mg/dL. Treatment with 4 g of pH-dependent release mesalamine (Asacol®) was started, and a significant clinical improvement was noted, with resolution of the bloody diarrhea and passage of 2 normal stools per day. Ten days later, while the patient was doing well, he presented with a 39 °C fever with rigor, myalgia and anorexia. Upon admission, 6 d after the fever onset, his physical examination was normal.

A blood analysis showed a hemoglobin level of 12.2 g/dL, a white blood cell count of 8600/mL (of which 77% were granulocytes), a platelet count of 264 000/mL, and a CRP level of 104 mg/dL, with normal levels of liver and pancreatic enzymes. Serologies for typhoid fever, brucellosis, parvovirus, Epstein Barr virus and cytomegalovirus were negative. Stool, blood and urine cultures were also negative. The tuberculosis skin test was normal as well as the chest X-ray and cardiac ultrasound. The mesalamine was discontinued, and the fever was treated symptomatically with paracetamol. Two days later, the patient's clinical condition improved, with the disappearance of the febrile syndrome and a decrease in the CRP level to 62 mg/dL. After discussing the matter with the patient and because a reactivation of the colitis was feared, particularly after the rapid remission that was obtained with the use of 5-aminosalicylic acid (5-ASA), we decided to reintroduce another time-dependent release mesalamine (Pentasa®) under medical surveillance. After administering the second dose, the patient presented with a 38.5 °C fever with rigors. Mesalamine was stopped, and the fever resolved the following day without any intervention. The diagnosis of fever related to mesalamine was confirmed. Two days later, the patient presented with bloody diarrhea, and a treatment with 40 mg of prednisone and 150 mg of azathioprine was started, with rapid improvement. The patient was discharged in good condition. Two months later, the prednisone was stopped after tapering, and the patient was maintained in clinical remission with azathioprine. Eight months later, the patient presented with a flare-up of his colitis. He consulted another gastroenterologist, who treated him with suppositories of Asacol because the rectosigmoidoscopy showed an active proctitis. Two days later, the patient presented with the same symptoms encountered with oral mesalamine (*i.e.*, fever and rigors), which spontaneously resolved after stopping the topical treatment.

## DISCUSSION

The 5-ASA agents are known to be safe and remain among the first-line approaches for inducing remission and preventing relapse in UC<sup>[1]</sup>. These benefits of 5-ASA agents are less impressive in the treatment of mild to moderate Crohn's disease. Their possible role in chemo-

prevention of colon cancer strengthens the indication for the long-term use of 5-ASA<sup>[2]</sup>.

The efficacy of sulfasalazine in UC has been known since 1940, but the use of this drug was problematic because of its side effects, either dose related or idiosyncratic reactions, such as headache, dyspepsia, nausea, hypersensitivity rash, hemolytic or aplastic anemia, and pulmonary or hepatic dysfunction<sup>[3]</sup>. The identification in sulfasalazine of the 5-ASA moiety, which is responsible for the therapeutic effects, led to the development of new drugs that delivered 5-ASA directly to the colonic mucosa with fewer side effects.

These new drugs included mesalamine or 5-ASA itself, olsalazine (Dipentum; a dimer of 5-ASA) and balsalazide (Colazal or Colazide; a pro-drug of 5-ASA). In their systematic review of the safety of 5-ASA-based agents, Loftus *et al*<sup>[3]</sup> concluded that all of these agents appeared to be safe and that the fraction of patients experiencing adverse events was similar to that seen in patients treated with placebo.

However, severe adverse events encountered with mesalamine therapy have been reported as isolated cases. A French pharmacovigilance study of mesalamine microgranules (Pentasa) reported between 6.6 and 9.0 adverse events per million treatment days over a 2-year period, including cases of pancreatitis, hepatitis, pericarditis and hematological disturbances<sup>[4,5]</sup>. Other side effects have also been reported, such as worsening colitis, renal toxicity (interstitial nephritis and nephrotic syndrome), pulmonary toxicity (interstitial lung disease and fibrosis, bronchiolitis obliterans, pulmonary granulomatosis and eosinophilic pleural effusion) hair loss and Stevens-Johnson syndrome<sup>[6]</sup>. Some of these drug-related complications must be distinguished from the extraintestinal manifestations of IBD, and they may arise independently of disease activity. The causal relationship between mesalamine administration and the occurrence of the adverse event is established by the appearance of the symptoms after the administration of the 5-ASA compound, the rapid resolution of the symptoms after discontinuation and the reoccurrence of the same symptoms if the treatment is restarted<sup>[6]</sup>.

The mechanism by which mesalamine induces side effects remains unclear. In some cases, the occurrence of a complication seems to be dose related, whereas in other cases it appears to be more of an idiosyncratic reaction<sup>[7]</sup>. Moreover, the pathogenesis of the fever induced by mesalamine remains unknown.

In cases of acute pancreatitis, the suggested mechanism is an increased permeability of the pancreatic duct directly due to the effects of salicylic acid<sup>[8]</sup>. In cases of mesalamine-induced exacerbation of UC<sup>[9]</sup> or myocarditis<sup>[10]</sup>, eosinophilic infiltration of the colon and the myocardium point to an allergic drug reaction.

Mesalamine-related complications are known to occur with oral and topical preparations. Some reports have claimed an absence of cross reactivity among different 5-ASA-based drugs<sup>[11]</sup>.

Our patient presented with an isolated fever while his bloody diarrhea was improving. Mesalamine-induced isolated fever has been reported by Gonzalo *et al*<sup>[12]</sup> and the causal relationship was proved by a placebo-controlled challenge test and a protocol of desensitization, which was realized successfully. We realized, under medical surveillance, a challenge test with a different 5-ASA drug, and the fever reappeared earlier than the first time (1 d *vs* 10 d). On both occasions, no other causes of fever could be identified, and the fever disappeared spontaneously after stopping the drug. Moreover, reintroducing the mesalamine in suppository form one year later led to the same side effect: an isolated fever that also resolved after stopping the treatment. Although mesalamine seemed to be efficacious, we did not choose to adopt a desensitization protocol at that time because the patient feared a more severe reaction, and we needed another strategy to treat the reactivated colitis.

In conclusion, mesalamine appears to be safe and to achieve good results in IBD patients, particularly those with UC. Side effects of 5-ASA-based drugs are rare, but they should be distinguished from extraintestinal manifestations and should be suspected when the symptoms resolve with the discontinuation of the treatment and reappear with its reintroduction. An isolated fever can reappear on multiple occasions after the reintroduction of various formulations of mesalamine, as illustrated in our case report.

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## Management of duodenal ulcer bleeding resistant to endoscopy: Surgery is dead!

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### Abstract

Acute massive duodenal bleeding is one of the most frequent complications of peptic ulcer disease. Endoscopy is the first-line method for diagnosing and treating actively bleeding peptic ulcers because its success rate is high. Of the small group of patients whose bleeding fails to respond to endoscopic therapy, increasingly the majority is referred for embolotherapy. Indeed, advances in catheter-based techniques and newer embolic agents, as well as recognition of the effectiveness of minimally invasive treatment options, have expanded the role of interventional radiology in the management of hemorrhage from peptic ulcers over the past decade. Embolization may be effective for even the most gravely ill patients for whom surgery is not a viable option, even when extravasation is not visualized by angiography. However, it seems that careful selection of the embolic agents according to the bleeding vessel may play a role in a successful outcome. The role of the surgeon in this clinical sphere is dramatically diminishing and will certainly continue to diminish in ensuing years, surgery being typically reserved for patients whose bleeding failed to respond all previous treatments. Such a setting has become extremely rare.

### TO THE EDITOR

We read with great interest the recent article by Wang *et al*<sup>[1]</sup> published in the September issue of the *World Journal of Gastroenterology* evaluating the efficacy and safety of emergency transcatheter arterial embolization (TAE) for patients with acute massive duodenal ulcer hemorrhage. We have several comments and questions.

Transcatheter embolization is now accepted as the salvage treatment of choice for acute bleeding from gastroduodenal ulcers. Many published studies have confirmed the feasibility of this approach and the high technical and clinical success rates, ranging from 91% to 100% and from 63% to 100%, respectively, in all case-series including more than 10 patients over the last decade<sup>[2,3]</sup>.

First, we are surprised in the present study that 19 (65.5%) of the 29 patients had no endoscopic hemostasis prior to TAE. In our experience, endoscopic therapy remains the first treatment modality in the management of bleeding peptic ulcers, even in those presenting with massive bleeding. On the other hand, it seems that the authors performed TAE in the gastroduodenal artery territory in 3 patients who did not undergo preliminary endoscopy and for whom angiography was negative. Does it mean that TAE was carried out in these patients without neither endoscopic nor angiographic data? Could the authors clarify this point? Indeed, several pre-

vious studies found that empiric embolization based on endoscopic findings, in the absence of contrast extravasation, was helpful in achieving bleeding control, with no difference according to whether angiography identified the bleeding site<sup>[4,5]</sup>. However, accurate endoscopic localization of the bleeding site is a prerequisite to allow empiric embolization for angiographically negative upper gastrointestinal bleeding.

Second, we would like to congratulate the authors on their high clinical success rate of 93% (27 of the 29 patients). However, we are surprised that these results were obtained with the use of sponge particles as the only embolic agent. Although the influence of the type of embolic agent on the clinical outcome is controversial, several authors reported a high rate of bleeding recurrence when gelfoam was used alone<sup>[6,7]</sup>, whereas the clinical success was relatively high in recent series in which glue was used as the only embolic agent<sup>[8,9]</sup>. In addition, two studies demonstrated a statistically significant association between the use of coils as the only embolic agent and greater rebleeding rates<sup>[2,10]</sup>. On the other hand, good results were reported with the combination of gelatin sponge and coils<sup>[2,11]</sup>. Based on our experience and the literature, we do not recommend the use of coils alone but in combination with gelfoam or glue, when using the sandwich technique in areas with rich collaterals like the gastroduodenal artery territory<sup>[2,10]</sup>. It allows a faster and better hemostasis, especially in patients with coagulopathy.

In addition, the normal collateral pathways after a successful embolization should be systematically checked to avoid retrograde filling through anastomoses as the inferior pancreaticoduodenal artery (IPDA) from the superior mesenteric artery in order to maximize results. Indeed, one explanation for good clinical results in this study might be the systematic use of this technique. It may be worthwhile for readers to know the number of patients in whom additional TAE of the IPDA was performed here. Another plausible explanation for high clinical success rate in this study could be the young age of the study population (36 years), without comorbidities. We know that underlying conditions can contribute to a poor outcome.

In conclusion, we agree with the authors about the safety and efficacy of TAE for the treatment of acute hemorrhage from duodenal ulcers. However, angiography should be performed only after failure of endoscopic hemostasis in such a setting. In most cases, embolization obviates the need for surgery and is associated with lower complications and mortality rates than surgical

hemostasis. Although prospective studies are needed to compare these management strategies, the available data suggest that TAE is a good alternative to surgery and could be considered the salvage treatment of choice after failed endoscopic treatment. The role of the surgeon in this clinical sphere will certainly continue to diminish in ensuing years.

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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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- 5 **Vallancien G**, Emberton M, Harving N, van Moorseelaar RJ, Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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

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DOI:10.1046/j.1526-4610.42.s2.7.x]

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- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

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

### Books

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- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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

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- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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